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Analysis of Abalone (Haliotis Discus Hannai and Haliotis Tuberculata) Shellfish Histology and Pathology Using Microscopic and Molecular Methods

Leanne Harris
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A thesis submitted for the degree of Doctor of Philosophy (PhD)

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ABSTRACT

Abalone is a marine univalve gastropod mollusc, which inhabits temperate and tropical waters in both hemispheres of the globe. Abalone shellfish are not endemic to Irish waters and were introduced into Ireland for commercial cultivation in the 1970s. Irish abalone farms have been successful since their establishment, producing 25 tonnes per annum. This success has had a knock-on effect with respect to increased interest in abalone shellfish research, both nationally and internationally. The aims of this research project were to study the biology of abalone through a histological characterisation of two species of this shellfish, *Haliotis tuberculata* and *Haliotis discus hannai* using both histochemical and immunohistochemical techniques and to develop molecular methods for the detection and quantitation of common shellfish pathogens associated with abalone disease outbreaks.

In the first part of this study immunohistochemistry was used to examine cell distribution, structural and functional proteins in abalone tissues. Phenotypic antigen expression was analysed in abalone shellfish using an array of antibodies that react with a range of proteins in many different species. The following antibodies reacted positively with antigens in abalone tissues: PCNA (Proliferating Cell Nuclear Antigen), cytokeratin MNF, cytokeratin AE1, vimentin, NSE (Neuron Specific Enolase) and laminin. Western blots were used to confirm the presence of proteins in abalone tissues. As there are currently no shellfish antibodies commercially available, antibodies that react to human and veterinary antigens were chosen and applied to abalone tissues to determine the extent of cross reaction between species and to identify the types of cell markers in these tissues.

In the second section of this study abalone functional biochemistry was characterised using both histochemical and enzyme histochemical staining methodologies. The distribution of both functional and structural elements such as enzymes, carbohydrates, lipids, pigments and minerals was investigated in abalone tissues. Enzymes such as acid and alkaline phosphatase, non-specific esterases, acetylcholinesterase, leucineaminopeptidase, carbonic anhydrase and peroxidase were detected in the cells of abalone tissues. Carbohydrates including glycogen, neutral mucins, acid mucins, sulphated acid mucins and carboxylated mucins were identified in abalone tissues. Lipids, pigments and minerals such as neutral lipids, copper, iron, melanin and calcium were also detected in the cells of abalone tissues. As there are very few histological and histochemical studies available for this shellfish genus, the aim of this section of work was to provide a more adequate and comprehensive study that acts as a basic biological reference for future studies on pathology.

The potential of infectious pathogens to cause high mortalities within shellfish populations has led to increased awareness of shellfish disease, along with the development of methods for the detection of pathogenic organisms. *Vibrio harveyi* (bacterium) and *Perkinsus olseni* (protozoan) are two major disease causing pathogens of abalone. In the final part of this study, nucleic acid-based methods for the detection and quantitation of *V. harveyi* and *P. olseni* were developed. A polymerase chain reaction (PCR) method was developed to detect DNA from isolates of *V. harveyi* and further developed into a novel multiplex PCR method, which simultaneously amplifies a 413 bp region of the 16S rRNA gene of *V. harveyi* and a 155 bp region of the actin mRNA gene of *Haliotis* spp. A PCR amplification method to detect *P. olseni* using novel primers (designed in this study) which amplify a 265 bp DNA fragment of the

ITS region of the genome of this organism was also developed. Both PCR methods were optimised for pathogen detection in alcohol preserved and paraffin embedded tissue samples. For rapid detection and quantitation of *V. harveyi* and *P. olseni*, two real-time SYBR Green PCR methods were developed.

This study has contributed information to the known functional and structural biology of abalone species. It has determined the distribution of structural and proliferative proteins such PCNA, cytokeratins, vimentin, NSE and laminin; established the enzyme profile of the various organs of abalone; and established the distribution of carbohydrates, lipids, pigments and minerals in abalone tissues. In addition molecular nucleic acid-based methods for pathogen detection in these shellfish have been developed, which may be of considerable value to the aquaculture industry both in Ireland and abroad.

DECLARATION OF WORK

I certify that this thesis which I now submit for examination for the award of Doctor of Philosophy (PhD), is entirely my own work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for an award in any other Institute or University.

The work reported on in this thesis conforms to the principles and requirements of the Institute's guidelines for ethics in research.

The Institute has permission to keep, to lend or to copy this thesis in whole or part, on condition that any such use of the material of the thesis be duly acknowledged.

Signature Leanne Harris Date 9.1.2007

Candidate

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Mam and Dad, you have never doubted me, have always believed in me, but most of all loved me. I would never have made it this far without you. I cannot begin to thank you enough. This thesis is dedicated to you both.

Éamonn, thank you for your love and support, you have been there for me throughout, you are my inspiration. I thank you!

'The ideal Scientist thinks like a Poet, works like a Clerk and writes like a Journalist'

E. O. Wilson

This thesis is dedicated to my Mam and Dad.

LIST OF ABBREVIATIONS

FULL TERM	ABBREVIATION
3-aminopropyltriethoxysilane	(APES)
5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside	(X-gal)
Alcian Blue	(AB)
Alcian Blue Periodic Acid Schiff	(ABPAS)
Aminopeptidase	(AMP)
Arbitrary Fragment Length Polymorphism	(AFLP)
Arg-Phe-amide	(RF-amide)
Avidin Biotin Complex	(ABC)
Base pair	(bp)
Bord Iascaigh Mhara	(BIM)
Bovine Serum Albumin	(BSA)
Central Nervous System	(CNS)
Cluster of Differentiation	(CD)
Colony Forming Unit	(CFU)
Deoxyribonucleic acid	(DNA)
Deoxyribonucleotide triphosphates	(dNTP)
Diaminobenzidine-peroxide	(DAB)
Diastase Periodic Acid Schiff	(DPAS)
Dimethyl sulfoxide	(DMSO)
Dipeptidyl aminopeptidase	(DAP)
Distrene Plasticiser Xylene	(DPX)
Enzyme Linked Immunosorbent Assay	(ELISA)
Epidermal Growth Factor	(EGF)
Extracellular matrix	(ECM)
Fluorescent *in situ* hybridisation	(FISH)
Food and Agriculture Organisation	(FAO)
Gamma-aminobutyric acid	(GABA)
Glial Fibrillary Acidic Protein	(GFAP)

Glial Filament Protein	(GFP)
Gram	(g)
Haematoxylin & Eosin	(H&E)
Highly Unsaturated Fatty Acid	(HUFA)
Horseradish peroxidase	(HRP)
Hour	(h)
Hydrogen potential	(pH)
Immunofluorescent assay	(IFA)
Immunoglobulin	(Ig)
In situ hybridisation	(ISH)
Insulin-like peptides	(ILP)
Intergenic spacer	(IGS)
Intermediate filament	(IF)
Intermediate Filament Antigen	(IFA)
Internal Transcribed Spacer	(ITS)
International Council for the Exploration of the Sea	(ICES)
Kilobase	(kb)
Kilodalton	(kDa)
Light Microscopy	(LM)
Litre	(l/L)
Luria Bertani	(LB)
Melt temperature	(Tm)
Microgram	(µg)
Microlitre	(µl)
Micromolar	(µM)
Micron	(µm)
Milligram	(mg)
Millilitre	(ml)
Millimolar	(mM)
Minimum Essential Medium	(MEM)
Minute	(min)
Muscle Specific Actin	(MSA)
Nanogram	(ng)
Neurofilament	(NF)

Neuron Specific Enolase	(NSE)
Nicotinamide dinucleotide	(NAD)
Nicotinamide dinucleotide phosphate	(NADP)
N,N-dimethyl formamide	(DMF)
Non Transcribed Spacer	(NTS)
Office International des Épizooties	(OIE)
Oil Red O	(ORO)
Optical Density	(OD)
Optimal Cutting Temperature	(OCT)
Periodic Acid Schiff	(PAS)
Peripheral Nervous System	(PNS)
Phe-Met-Arg-Phe-amide	(FMRF-amide)
Phosphate Buffered Saline	(PBS)
Polymerase Chain Reaction	(PCR)
Polyvinylidene difluoride	(PVDF)
Proliferating Cell Nuclear Antigen	(PCNA)
Ray's Fluid Thioglycollate Medium	(RFTM)
Relative Centrifugal Force	(g)/(RCF)
Relative Fluorescence Units	(RFU)
Research and Development	(R&D)
Restriction Fragment Length Polymorphism	(RFLP)
Revolutions per minute	(rpm)
Ribonucleic acid	(RNA)
Ribosomal ribonucleic acid	(rRNA)
Room temperature	(RT)
Small subunit	(SSU)
Sodium dodecyl sulphate	(SDS)
Sodium dodecyl sulphate-polyacrylamide gel electrophoresis	(SDS-PAGE)
Species (sing./pl.)	(Sp/Spp)
Super Optimal Catabolite Repression	(SOC)
Thermus aquaticus	(Taq)
Thiosulphate Citrate Bile Salts	(TCBS)
Top Working Standard	(T.W.S.)
Transmission Electron Microscopy	(TEM)

Tris Borate EDTA	(TBE)
Tris Buffered Saline	(TBS)
Tris EDTA	(TE)
UltraViolet	(UV)
Unit	(U)
Voges-Proskauer	(VP)
Von Willebrand Factor	(VWF)
Water	(H_2O)

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CHAPTER 1

GENERAL INTRODUCTION

1.1 AQUACULTURE

1.1.1 General Aquaculture

Aquaculture can be described as encompassing all activities aimed at producing, processing and marketing aquatic plants and animals from fresh, brackish and salt waters (Barnabé 1990). In simpler terms, it describes the farming of any aquatic species, the major taxonomic groups being fish, crustaceans, molluscs, seaweeds, microalgae, amphibians and reptiles (Bostock 2002). The Food and Agriculture Organisation (FAO) of the United Nations have defined aquaculture as the farming of aquatic organisms whereby farming implies some form of intervention in the rearing process to enhance production such as regular restocking, feeding and protection from predators (www.marine.ie). The broadest definition describes aquaculture as the large-scale husbandry or rearing of aquatic animals for commercial purposes (Landau 1992).

Aquaculture in the past dates back to prehistoric times, with evidence of fish culture in ancient Chinese manuscripts (Bardach et al. 1972) and in ancient Greece and Rome (Barnabé 1990). According to Boyle (1981) the origins of man's use of molluscs as food, are lost in prehistoric times as practically all of the coastal dwelling sites of Neolithic ages in western Europe, the Mediterranean, Malay Archipelago, Australasia and South America, show evidence of man's exploitation of locally available molluscs. Aquaculture has progressed rapidly since ancient times with the development of modern techniques that allow for the cultivation of more species, as well as opening up opportunities for the cultivation of new areas of water (Barnabé 1990). In the last fifty years, the average fish consumption per person has almost doubled in number and this boost in consumption has been made possible by the dramatic increase in farmed fish and shellfish production (Watson and Stokes 2003a; Gibbs 2004; Bell et al. 2006).

Global demand for seafood is increasing, however natural fish stocks can only supply a limited amount of food which means that sustainable aquaculture is becoming a vital source of food and resources. Aquaculture is one of the fastest expanding sectors of food production in the world with an average growth rate of 9.2% in the last fifteen years (Bostock 2002) and world total demand for fish and fishery products is expected to reach 183 million tonnes by 2015 (FAO 2004).

This global increase in aquaculture production is also very progressive, in that sustainable aquaculture can alleviate poverty through the provision of steady state employment (Watson and Stokes 2003a). Aquaculture of marine invertebrates is an economically important activity in both developed countries and developing countries (Mialhe et al. 1995). It is predicted that by the year 2030 less than 50% of global fish consumption will have originated from capture fisheries (fishing activity that involves the capture of wild fish or shellfish) and developing countries will be supplying an increasing amount of produce to developed countries (Bostock 2002; FAO 2004). Commercial aquaculture contributes significantly to the economies of many countries. Molluscan aquaculture represents a major global industry which accounted for 23% of global production and 16% of its value in 1998 (Bostock 2002). It is estimated by the FAO that fishery production will reach between 107 million and 144 million tonnes by 2010 and of that, it is proposed that between 74 and 144 million tonnes will be readily available for direct human consumption. The majority of this increase is expected to come from aquaculture which is envisaged to supply between 35 and 40 million tonnes (Bostock 2002; FAO 2004).

Aquaculture is an environmentally friendly industry, in that it aids in the prevention of the extinction of marine organisms by relieving the pressure on stocks of wild animals. In 1972, Bardach et al. described the increase in the production by world fisheries but noted that stocks of aquatic organisms are limited and eventually a ceiling will be reached on the harvest of wild aquatic animals. Since then the aquaculture industry has soared on a global scale. Globally, aquaculture is expanding faster in Africa and South America than anywhere else in terms of volume and value. Output from North America has remained constant while in Europe, aquaculture has declined in terms of value. In Asia, China remains the dominant producer in the aquaculture industry (Bostock 2002; FAO 2004).

1.1.2 Abalone Aquaculture

Records of the very first abalone fisheries date back to 1500 years ago in China (Nie 1992). In 1913 Ernest Doelter was the first to develop abalone as a domestic gourmet food item and in 1915 he introduced his newly found epicurean specialty to the world at the Panama Pacific International Exposition (Howorth 1978). Abalone aquaculture was established in Japan in the last fifty years and other Asian countries followed their lead, with China now the principle abalone producer in the world (Viana 2002). The well-developed muscular foot of abalone has long been in demand in the Orient with fisheries being developed worldwide in order to supply the market with this luxury food (Shepherd et al. 1992). As the fisheries decline, aquaculture ventures are attempting to meet the shortfall to satisfy market demand (Bostock 2002). South Africa has recently become the largest abalone producer outside of Asia followed by Australia and the USA (FAO 2004; Troell et al. 2006). Australia is the leading country in research for new grow-out technologies (in aquaculture 'grow-out' refers to the stage at which young fish

are grown to market size), as well as the development of specialised diets, and the introduction of novel abalone species for cultivation (Viana 2002; Daume 2006). Another indication of the success of the abalone industry in Australia has been the additional introduction of abalone pearl production (Viana 2002). Africa has surpassed the USA with six farms in production selling their products directly to Japan. The USA began abalone culture in the 1980s, however production has since declined. Other countries where this industry looks promising include Chile, New Zealand, Iceland, France and Ireland (Viana 2002).

The most recent update of abalone aquaculture was performed by Gordon and Cook (2004), in which they described the phenomenal growth of global cultured abalone in recent years. More than 15 species of abalone are being commercially cultivated on a global scale. The main abalone species being cultured are Japanese abalone (*Haliotis discus hannai*), disk abalone (*Haliotis discus discus*), red abalone (*Haliotis rufescens*), blacklip abalone (*Haliotis rubra*), European abalone (*Haliotis tuberculata*), Blackfoot abalone (*Haliotis iris*) and South African abalone (*Haliotis midae*). Other species are cultivated on a much smaller scale (Bostock 2002).

The abundance of abalone on the market can be attributed to the number of culture facilities available which are constantly increasing. Aquaculture facilities should ideally lead to sustainability through reduced pressure on wild populations, however there has been a massive increase in illegal take (any world harvest of abalone beyond the total allowable annual landing quotas) from the late 1990s to the present, which has offset the advantages of aquaculture (Gordon and Cook 2004).

1.1.3 The Irish Perspective

It is part of national fisheries policy in Ireland to promote the sustainable development of aquaculture through the expansion of fish and shellfish farming (Costello 2000). Since the early 1970s, Irish aquaculture has been a significant contributor to the Irish economy with increases in output, value and job creation. It has also made a vital contribution to local economies in remote coastal areas (Meldon 1993). Owing to a rapid growth in the industry in the 1980s, the output value from Irish aquaculture has increased from €37.2 (IR£29.3) million in 1990 to €101.5 million in 2003 (Parsons et al. 2004). Combined production from both finfish and shellfish aquaculture is expected to reach €175.6 million in 2008. Total production in the shellfish sector in 2003 was €41.8 million. No doubt the success story of Irish fish farming has been the Atlantic salmon (*Salmo salar*) with output rising from virtually nothing in 1970 to the current output level of approximately 15,000 tonnes per annum (Parsons et al. 2004). While shellfish production is not as extensive as finfish production, it is a significant industry and makes a considerable contribution to the economy. It is for this reason that new species of shellfish have been introduced into Ireland in the last few years. Irish farmers have had to expand and diversify in response to the increasing consumer demand for fish and shellfish (Watson and Stokes 2003a). The Department of the Marine and Natural Resources and Bord Iascaigh Mhara (BIM) jointly launched a €72.4 (IR£57) million investment plan for the aquaculture sector for the period 2000-2006, which aims to see an increase in the value of aquaculture (Watson and Stokes 2003a; Parsons et al. 2004). The main shellfish species under cultivation in Ireland include Pacific oysters, native flat oysters, clams, scallops and mussels which account for 80-90% of annual shellfish production. Abalone and sea urchins are produced on a much smaller scale, however,

steady progress was made in 2003 with abalone production moving progressively towards a full-scale commercial level (Parsons et al. 2004).

Commercial cultivation of abalone shellfish in Ireland began in the 1970s. Abalone is not native to Ireland so hatcheries and cultivation techniques were developed for two species of this shellfish. The European abalone *Haliotis tuberculata* otherwise known as the ormer, was brought from Guernsey and the Japanese abalone *Haliotis discus hannai*, more commonly referred to as ezo awabi, was introduced from Japan (La Touche and Moylan 1984; La Touche 1986). Ireland is in an unrivalled position in Europe in having these two species of abalone in culture systems (Watson and Stokes 2003a; Huchette and Clavier 2004). These shellfish are cultivated using land-based or marine-based methods that operate according to the growing techniques employed in Australia, China, Taiwan and the USA (Watson and Stokes 2003a). Pilot scale trials established that it was possible to cultivate these shellfish here and private hatcheries have since been developing in a number of areas around the west and south west coasts of Ireland e.g. Ballinakilla Bere Island, Cork; Clifden, Galway; Letterfrack, Galway; Castlegregory, Kerry; Belmullet, Mayo and Westport, Mayo (DEFRA 2001).

Initially Irish farmers had a number of challenges to face with the introduction of these two foreign species. Commercially important abalone species usually prefer warm temperatures ranging from 15°C-30°C. *Haliotis discus hannai* is a cold water mollusc and can grow 10% faster than its European counterpart in Irish waters. Due to the differences in growth rates between these two abalone species, growing conditions were optimised through the implementation of temperature regulated hatcheries. Another major difference between the two species is their feeding preferences. *Haliotis*

tuberculata favours red seaweed and dillisk to the common Irish brown seaweeds and kelps, which in the beginning posed problems for Irish farmers (La Touche 1986). The Japanese abalone is content to feed on kelp but feeding on other weeds also enhances its growth rate. The mortality rate is thus higher amongst European abalone populations in comparison with the Japanese abalone. Despite the initial difficulties for Irish farmers, the market for abalone shellfish is undersupplied, with demand almost always exceeding supply, presenting the opportunity for profitability. *Haliotis tuberculata* can command a good price in European markets but has yet to be tested in South East Asian markets. *Haliotis discus hannai* is much sought after by Japanese communities as it makes up to 46% of the wild catch and the majority of cultured abalone seed in Japan. It thus gives Irish farmers a significant opportunity to supply this product to the Japanese community (approximately 150,000 people) in the European marketplace (Watson and Stokes 2003a; www.jref.com).

As previously mentioned there are six abalone ongrowing units in Ireland with spat output from these hatcheries approaching one million shellfish per annum. It was expected that the overall production of animals for the country was set to reach 25 tonnes by 2005 (Watson and Stokes 2003a). This is small scale production in comparison with China which currently operates over 300 farms with a total annual production of 3,500 tonnes (Watson and Stokes 2003a). However, the success to date of these shellfish farms in Ireland and the popularity of these new species with producers, is very promising for the fishing industry, the marine environment and the Irish economy.

1.2 ABALONE

1.2.1 Phylum Mollusca, Class Gastropoda

Brusca and Brusca (2003) have referred to the mollusca as one of the best known groups of invertebrates as everyone is familiar with snails, clams, slugs, squid and octopus. The number of described species of living molluscs is estimated to be in the region of 100,000 along with 60,000 fossil species (Brusca and Brusca 2003). Molluscs have always been highly valued because of their usefulness as tools, implements, money and most importantly as a food source.

Abalone is a member of the Class Gastropoda which is the largest and most successful groups of molluscs (Ruppert and Barnes 1994). All abalone belong to the Family Haliotidae and its Latin genus name *Haliotis*, was assigned by Linnaeus in 1740 and translates into "sea-ear" in reference to the ear-shaped shell of this mollusc (Crofts 1929). Abalone is a marine univalve or single-shelled mollusc, a common feature of the gastropods (Mgaya and Mercer 1994). It can also be classified further into the Subclass Prosobranchia which describes all gastropods in which the mantle cavity, gills and anus are located at the anterior of the body so torsion is quite distinct and gills carry out respiratory activities. A nocturnal forager, abalone is a relatively primitive prosobranch and is often referred to as an archaeogastropod as it has changed very little since its first emergence, millions of years ago. Fossil abalone have been found from the late Cretaceous through the late Pleistocene, with a conspicuous hiatus in the early Paleogene (Geiger and Groves 1999). The oldest known abalone species in the world is *Haliotis lomaënsis* which lived in California in the Upper Cretaceous Period. This fossil is eighty million years old, measures a half-inch in length and differs very little from its modern counterpart (Howorth 1978; Geiger and Groves 1999).

Members of the family Haliotidae are found distributed throughout the temperate and tropical waters of both hemispheres of the globe (Geiger 2000). According to Geiger (2000) there are 56 described species of abalone of worldwide distribution. Numerous different species are found in the tropical western Pacific, Australia, Japan, South Africa and along the coast of the northeastern Pacific margin (Lindberg 1992). There are no abalone species of global distribution, with the largest areas inhabited by some Indo-Pacific taxa such as *Haliotis clathrata*, *Haliotis asinina*, *Haliotis planata* and *Haliotis ovina*. All other species have more restricted ranges (Geiger 2000).

1.2.2 *Haliotis discus hannai* and *Haliotis tuberculata*

Haliotis discus hannai and *Haliotis tuberculata* are two species of abalone that are cultivated in Ireland quite successfully (Fig. 1.1, Fig. 1.2). There are some noticeable differences between these two species, especially with respect to their natural environments, feeding habits, external appearance, their geographic ranges and habitats, but their basic life cycle is similar to most other abalone species.

Figure 1.1. ***Haliotis discus hannai*** (Taken From: www.conicyt.cl).

Figure 1.2. ***Haliotis tuberculata*** (Taken From: www.glaucus.org.uk).

The Japanese abalone *Haliotis discus hannai* (Fig. 1.1) naturally occurs in Dalian City and Changhai County in Liaoning Province, in Changdao County and Qingdao City and its counties in Shangdong Province (Nie 1992). The habitat of *Haliotis discus hannai* is rocky bottom, with juveniles in shallow water near low water mark, and adults in water from 1.5-12 m in depth (Nie 1992). This species feeds mainly on brown algae and sexual maturity is reached when both sexes reach 60 mm shell length. The shell of *Haliotis discus hannai* is highly ornate with a row of raised respiratory pores and a high spire. Externally the shell is green in colour and the inside of the shell is lined with mother of pearl. This abalone species can withstand low temperatures in comparison with the European abalone which prefers warmer waters, and the temperature for maximum growth rate is 20°C (Mgaya and Mercer 1994).

The European abalone *Haliotis tuberculata* (Fig. 1.2) is the only species in the family Haliotidae that is commercially harvested in Europe. It has a geographic range that extends in the eastern Atlantic from the Channel Isles and the French coasts of the Western Channel in the north, to the Cape Verde Islands and the West African coasts of Mauritania and Senegal in the south (Mgaya and Mercer 1994). The ormer prefers sublittoral rocky habitats with fissures and crevices in the bedrock (Mgaya and Mercer 1994). It feeds on seaweeds, algae and kelp but has preferences for red over brown seaweed. The colour of its shell varies from a mottled greyish green to red, depending upon the rock type in its surroundings and its algal diet (Mgaya 1995). The oval ear-shaped shell is slightly coiled with a low spire and a flattened whorl at one end and inside the shell is lined with mother of pearl as is the case with all abalone species.

1.2.3 Abalone Life Cycle

Abalone are dioecious or gonochoristic organisms meaning that sexes are separate and fertilisation is an external event. Fig. 1.3 illustrates the stages in the life cycle of abalone.

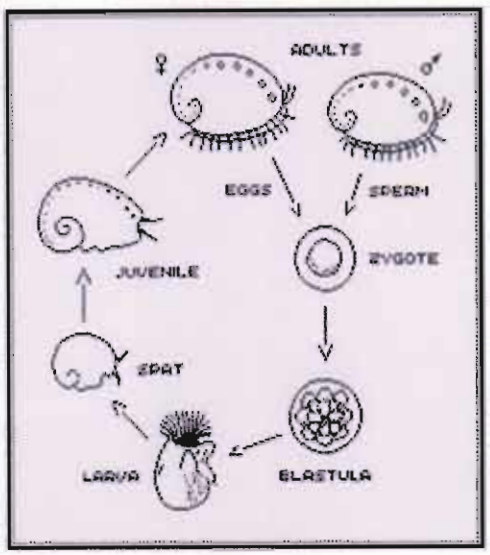

Figure 1.3. The Life Cycle of Abalone (Taken From: www.ingvar.is).

Abalone have a seasonal reproductive cycle consisting of an annual spawning preceded by gametogenesis (McShane 1992). Male and female abalone can be distinguished by the colour of the reproductive organ. When the gonad is ripe in males it appears cream in colour as opposed to the female gonad which is dark green. Spawning amongst the Haliotidae usually occurs in late summer (Crofts 1929). The fecundity of the various species of abalone differs, but in general a female can produce millions of eggs (Newman 1967; McShane 1992). The animal draws the shell down which applies pressure to the visceral mass aiding in gamete release. In brief, the contents of the gonad empty into a gonoduct that connects the gonad to the right kidney. The gametes escape into the branchial chamber through the right renal opening. The method of spawning is the same for both sexes whereby millions of gametes are released through the

respiratory pores out into the sea which is often known as broadcast or free spawning (Stephenson 1924; Bevelander 1988).

Abalone development has four stages (Bevelander 1988): fertilisation; cleavage of the fertilised egg which gives rise to differentiated cells; gastrulation which initiates organ formation and maturation whereby the animal assumes adult characteristics. As broadcast spawners, fertilisation for abalone can be relatively haphazard as it is only when the sperm and ova come into contact with each other that fertilisation can take place (Bevelander 1988). The eggs usually hatch within twenty four hours of fertilisation (Mottet 1978). After fertilisation a free-living upward swimming trochophore larva forms which is most likely an adaptation to avoid predators (McShane 1992; Ruppert and Barnes 1994). The veliger larva develops which is a downward swimming larva. The larvae swim by means of cilial beating, which does not allow for good directional movement (McShane 1992). The larvae remain planktonic for several days until an appropriate substrate is found for settlement which leads to metamorphosis from the pelagic to the benthic form. Lecithotrophic larvae are maintained on egg yolk when in the veliger form but begin to eat diatoms and microalgae when they become benthic organisms (Bevelander 1988). For lecithotrophic species such as abalone, successful settlement depends on competent larvae making contact with suitable substrata before the larvae starve to death. In their natural environment abalone tend to settle exclusively on coralline algae as the cavities within the coral provide shelter and protection for the relatively vulnerable larvae (McShane 1992). The larvae then shed their swimming hairs or cilia and begins formation of the adult shell. Survival to adulthood is reliant upon the suitability of the environment and as a result mortality amongst these animals is quite high.

1.3 ABALONE PHYSIOLOGY, HISTOLOGY AND PATHOLOGY

The shell houses the internal organs of the abalone shellfish. The spire is located posteriorly and the cephalic tentacles and eyes protrude from the anterior region of the shell. The epipodium extends out from the foot which is beneath the shell. The buccal mass occupies the central area at the anterior end which joins up with the oesophagus, crop, stomach and intestine that run posteriorly down the left side of the animal (Fig. 1.4). The hypobranchial gland and gills are found below and to the left of the buccal mass. The digestive diverticulum and gonad occupy most of the posterior region of the abalone with the rectum and heart residing to the left of the digestive diverticulum (Bevelander 1988; Mgaya 1995).

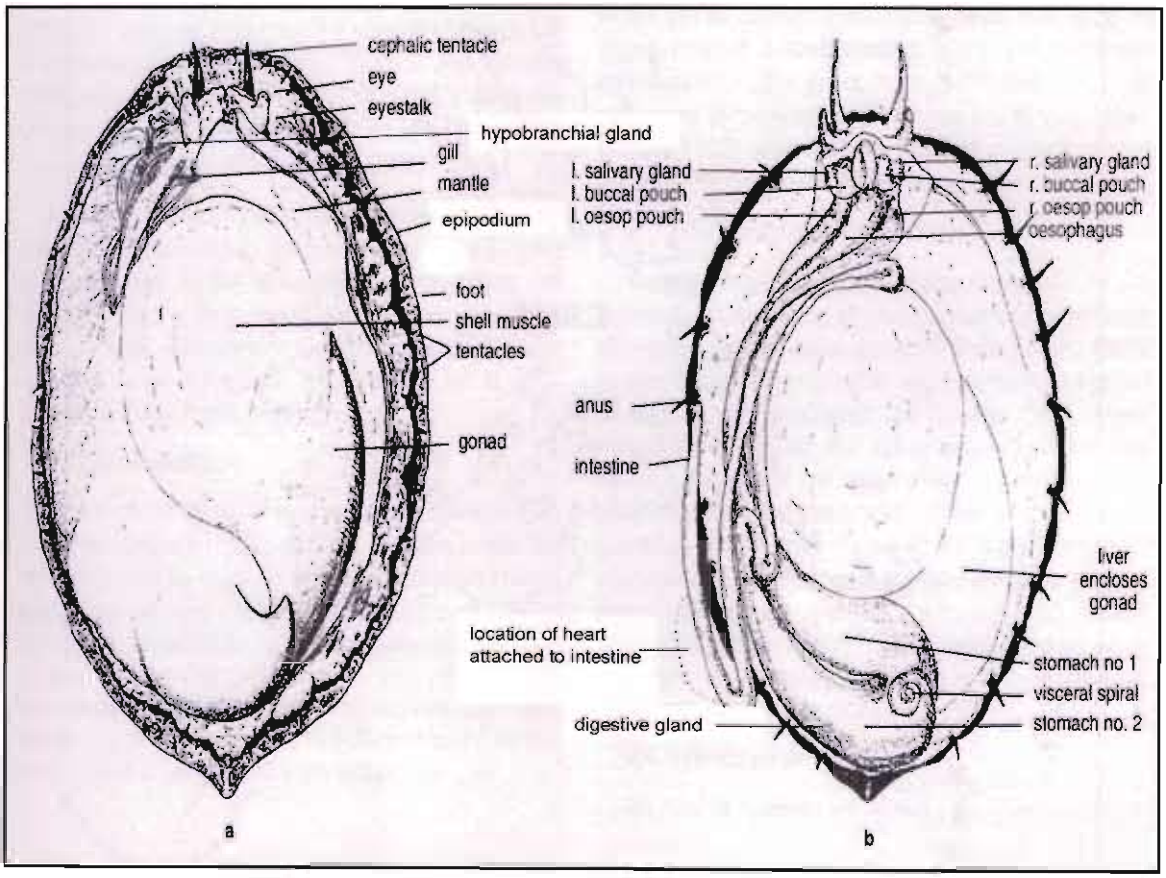

Figure 1.4. Anatomy of Abalone (Adapted From: Mgaya, Y. D. 1995. Synopsis of Biological Data on the European Abalone (ormer), *Haliotis tuberculata* Linnaeus, 1758 (Gastropoda: Haliotidae). FAO Fisheries Synopsis. No. 156. Rome. 1-28).

1.3.1 External Features

Shell

Abalone is rather primitive with a basic physiology and anatomy (Fig. 1.4). Abalone bears a single ornate calcium carbonate shell which is formed and secreted by the mantle (Sud et al. 2002). The shell is ear-shaped, with a posterior spire from which the respiratory pores begin and which are aligned along the left side of the shell (Bevelander 1988). As the shell increases in size a pearly substance is secreted from the mantle that closes the pores of the shell. Only four to five pores remain open at any time and they function in respiration, the excretion of waste material and spawning. Striations on the dorsal surface of the shell are called "growth rings" and form as a result of changes in the external environment such as water temperature, food availability or spawning periods (Bevelander 1988). The entire inner surface of the shell is iridescent in appearance as it is composed of nacre which is more universally known as "Mother of Pearl" (Giles et al. 1995).

Foot

Another conspicuous external feature of abalone is the foot. It is a large muscular mass with an extensive nerve and vascular supply rendering it a sensory and locomotory organ (Bevelander 1988). The foot forms an extensive ventral creeping sole which gives it a large surface area for adhesion (Crofts 1929). The sole of the foot is ciliated and extremely glandular. The gland cells produce copious amounts of mucus which, in conjunction with waves of muscular contraction, allow for movement over substrata (Brusca and Brusca 2003). The gastropod foot is also involved in many other functions such as locating and manipulating food, attaching eggs to substrates (this does not apply to abalone shellfish which are broadcast spawners), cleaning the shell, finding potential

mates and thwarting predators (Voltzow 1994). The foot is composed of epithelial tissue and connective tissue but it is primarily muscular (Voltzow 1994). Stratified columnar epithelium underlain by a basement membrane lines the sole of the foot and a single layer of cuboidal epithelium is found around the periphery of the foot. Individual muscle fibres are spindle shaped, unicellular and disorganised with respect to thick and thin filaments. Muscle type is chiefly smooth surrounded by a connective tissue sheath of collagen (Voltzow 1994). Associated with the sole of the foot are mucous cells, sub-epithelial glands and sub-epithelial ganglion cells. Gland cells and mucus producing cells are thought to aid in locomotion and adhesion (Voltzow 1994).

Mantle

The mantle is a thin sheet of connective tissue that houses the internal organs of abalone. It is involved in the production of both the mineral and organic components of the shell as well as functioning as an accessory respiratory organ. This sheet of loose connective tissue contains muscle fibres and a rich supply of nervous and vascular elements covered by epithelium. The epithelium of the mantle is either columnar or squamous but the cells thicken and deepen at the edge of the mantle as it becomes folded and pleated. Mucous cells, ciliated and non-ciliated epithelial cells are found throughout the mantle (Bevelander 1988).

Epipodium

The epipodium is a dorso-lateral development of the foot and is primarily sensory in function. When the epipodium is fully outstretched it can receive signals from a substantial range of the surrounding area (Crofts 1929). As well as being sensory in function the epipodium is thought to be involved in orientating and cleaning the shell as

the tentacles constantly sweep over the shell (Crofts 1929). The epipodium has an extensive nervous system extending into the tentacles which are covered in papillae increasing their sensitive surface area. The cells that make up this organ include sensory and supporting epithelial cells, sub-epithelial nerve cells and mucous cells. The sensory cells are spindle shaped epithelial cells, while the support cells are columnar in shape. Connective tissue is found underlying the epithelial cells (Crofts 1929).

1.3.2 Internal Features

The Digestive System

The digestive system is a continuous tube beginning anteriorly with a ventral mouth or buccal region which consists of a short snout, odontophore and buccal cavity (Bevelander 1988). The odontophore is a large muscular mass that supports and controls the movements of the radula, a scraping organ used to remove food particles from the substrata for ingestion (Brusca and Brusca 2003). The radula is a flexible chitinous band bearing transverse rows of teeth (Voltzow 1994). The digestive system also encompasses the jaws, salivary glands, oesophagus, crop, stomach, caecum, intestine, digestive diverticulum and a rectum ending in the mantle cavity with an anal opening (Bevelander 1988; Voltzow 1994).

The salivary glands are tubulo-alveolar glands and are found at the borders of the buccal mass. Histologically they are made up of ciliated glandular epithelial cells interspersed with goblet cells (Crofts 1929). The prosobranch oesophagus consists of three main regions, anterior, middle and posterior regions (Graham 1932; Bevelander 1988; Voltzow 1994). The anterior oesophagus has longitudinal folds that are separated by a groove called the dorsal food channel and it extends from the radula to the oesophageal

pouches; the middle section extends to the posterior portion of the oesophagus which is where the third portion begins and continues to the stomach (Bevelander 1988; Voltzow 1994). The ventral oesophageal wall has numerous gutters and ridges while the dorsal wall has irregular curved pleats lined with numerous papillae. The epithelial cells of the oesophagus are usually ciliated columnar cells. The oesophageal pouches are thrown into folds, contain specialised cells and are also covered in papillae (Bevelander 1988).

The oesophagus widens at a point where it becomes the crop (stomach 1 in Fig. 1.4). The crop is often described as the first part of the stomach and lies ventrally in the visceral mass (Crofts 1929; Bevelander 1988). A semicircular valve guards the opening of the crop and at the posterior end a narrow opening continues as the second part of the stomach (Bevelander 1988). Ciliated, secretory and glandular cells make up the columnar epithelium of the crop. The second part of the stomach (stomach 2 in Fig. 1.4) extends from the distal part of the crop to the intestine at which point the stomach bends at an angle of 180°. The cells lining the stomach appear columnar and rest upon a basal lamina (Bevelander 1988). The surface of these cells is covered with a fibrous sheet known as the gastric shield. The stomach proper has a spiral caecum or pouch-like extension consisting of columnar epithelial cells (Bevelander 1988). A valve connects the stomach to the intestine of abalone at which point the gastric shield disappears. A double fold called the typhlosole extends longitudinally throughout the intestine and caecum, dividing the caecum into two separate grooves. The caecum functions in the storage of digestive juices (Crofts 1929). The extended length of the intestine allows for efficient digestion and absorption. It becomes convoluted bending back upon itself forming many loops ending in the rectum. The whole of the intestinal columnar

epithelium has both ciliated and mucous cells which lie upon a lamina composed of scattered muscle fibres, connective tissue, fibroblasts, nervous and vascular elements.

The digestive diverticulum or hepatopancreas is a large gland taking up most of the visceral mass. It is made up of two types of secretory cells with ducts that transmit secretions to the caecum of the stomach. The duct cells are tall with an oval nucleus located in the lower one third of the cell. The cytoplasm of these cells contains numerous granules and vacuoles (Bevelander 1988). The crypt cells are smaller than the duct cells, relatively angular and are dark staining owing to the presence of iron granules. The digestive gland produces copious amounts of enzyme-containing fluids to aid in digestion. The entire digestive system is surrounded by a sheath of connective tissue that is lymphoid in nature and is well vascularised (Bevelander 1988).

The Excretory System

Abalone belongs to the Diotocardia, a division of the Prosobranchia and therefore has two excretory organs, both right and left kidneys (Voltzow 1994). The kidneys differ both structurally and functionally. The kidneys are located anteriorly in the visceral sac, beside the pericardium and covered by the digestive diverticulum. The renal cavity narrows beside the pericardium and urine empties into the mantle cavity by means of a small opening in the posterior roof to the right of the basibranchial sinus. The right kidney functions primarily in nitrogenous excretion and resorption and the left kidney is solely the site of organic solute and ion resorption (Andrews 1985; Voltzow 1994). The right kidney is embedded in lymphoid tissue and is composed of secretory epithelium that is squamous or cuboidal in shape unlike the left renal organ which has ciliated irregular cuboidal epithelium. Secondary lysosomes (containing enzymes), coated pits

and vesicles are abundant in the left kidney, indicating its role in resorption (Bevelander 1988).

The Circulatory System

The circulatory system includes a three chambered heart that distributes blood to various parts of this organism through arteries that terminate in veins and sinuses (Bevelander 1988). Abalone have two auricles and one ventricle and the heart is displaced to the left of the body (Voltzow 1994). The ventricle is more muscular and has thicker walls than the auricle. All three heart chambers have an epicardium lying on a basal lamina which overlies a myocardium (Voltzow 1994). The ventricle is comprised of branching muscle fibres and the posterior region of this cavity is attached to the intestine by muscle fibres. The auricles are large terminations of the gill vessels and their walls are very thin. They consist of delicate strands of muscle, connective tissue and epithelium. Large blood vessels are lined with endothelium surrounded by a thin connective tissue layer, but smaller vessels and sinuses lack these components. Gastropod circulatory systems are often referred to as 'open' systems as blood moves from arteries to large open spaces before returning to the heart (Hyman 1967; Voltzow 1994). These spaces are visceral, cephalic or cephalopedal sinuses so there are essentially no capillaries to bridge the gap between the arterial and venous systems rendering it an 'open' system. The heart receives oxygenated blood from the respiratory organs (gills and mantle) and pumps it to the various organs of the body. Hemolymph or blood is found in the body cavity which is called the hemocoel (Voltzow 1994). The hemolymph of *Haliotis* has a bluish tinge due to the presence of the oxygen carrier hemocyanin (Bevelander 1988). The bloods cells are leukocytic as identified by Crofts (1929) and are thought to have phagocytic properties in bivalve defence (Carballal et al.

1997; Wootton and Pipe 2003). Three main types of hemocytes exist, granular hemocytes called granulocytes and hemocytes with few granules or lacking granules referred to as agranulocytes and hyalinocytes (Voltzow 1994; Bachère et al. 1995).

The Respiratory System

The gills or ctenidia are specialised respiratory organs in *Haliotis*, however the mantle is also thought to be involved in respiration (Crofts 1929). The left and right gills are composed of bilaterally arranged leaflets (lamellae) that arise from a central axis or rachis. The left gill is always slightly larger than the right. The ventrally located skeletal rods are V-shaped and enclose the efferent lacunae. The rods provide basal support and maintain the lamellae in an erect position which aids in the free circulation of water between them. The gills are involved in gaseous exchange, blood transport and water movement (Bevelander 1988). The surfaces of the gill lamellae are separated by strands of connective tissue beneath a columnar epithelium interspersed with mucous cells. There are squamous epithelial cells in the convoluted regions that are most likely involved in oxygen and carbon dioxide exchange. The cells have both cilia and microvilli, indicating their role in active transport (Bevelander 1988). Each epithelium rests upon a basal lamina, muscle fibres, nervous and vascular elements which separate the lamellar surfaces. Where the gill axis joins the mantle skirt (at the anterior end of the gill axis) there are two structures called osphradia that function in assessing water quality prior to oxygenation of blood by the gills. The surface of the osphradia is lined with epithelium of two kinds, a single glandular layer and a stratified sensory layer. The osphradial surface is highly innervated. Exposure of the osphradia to toxicity of any type results in the closure of the respiratory chamber (Bevelander 1988). The

hypobranchial gland lies beside the left gill, produces mucus and is comprised of mucous cells, muscle and connective tissue (Bevelander 1988).

The Nervous System

The typical prosobranch nervous system is streptoneurous, meaning that the cerebral ganglia, the pleuropedal ganglion and the visceral ganglion are crossed to form a characteristic figure of eight (Hyman 1967; Kruatrachue et al. 2002). In other words it is a series of flattened ganglia distributed on loops of string that spread to flat commisures and connectives (Crofts 1929; Voltzow 1994). In *Haliotis* the nervous system is very primitive and shows little concentration of elements (Crofts 1929; Bevelander 1988). The two cerebral ganglia lie on the buccal mass and merge into a commissure that connects them. Each cerebral ganglion is extended anteriorly and ventrally as a nerve trunk. Both trunks meet underneath the oral tube close to the mouth completing the circumoesophageal nerve ring (Bevelander 1988). Each cerebral ganglion bifurcates into nerve trunks that run posteriorly and ventrally to a large nerve mass in the anterior of the foot. The two ventral trunks join up with the left and right pedal ganglia that are more anteriorly located in the foot. Nerves from the cerebral ganglia extend to the eyes, statocysts, cephalic tentacles and the epithelial cells of the lips and proboscis, head and neck (Voltzow 1994). Nerves from the buccal ganglia pass to the walls of the pharynx, the muscles of the buccal mass, the salivary glands, the oesophagus, stomach and other visceral organs.

The Reproductive System

As previously stated (1.2.3 Abalone Life Cycle) *Haliotis* is a dioecious organism so it is in possession of a single gonad which is either male or female (Crofts 1929). The gonad

is a conspicuous organ forming a large part of the visceral mass. It is located posteriorly and to the right side of the body. It is referred to as an arborescent racemose gland that occupies a space between the external integument and the outer surface of the digestive diverticulum. The epithelia and the underlying connective tissue grow out to form trabeculae and the epithelial cells produce either sperm or ova (Bevelander 1988). The sperm at the optical level consists of a head, body and tail piece. The ova are spherical and consist of cytoplasm with embedded nuclei. The cytoplasm is enclosed by a vitelline layer separated from the edge of the ova by a vitelline space. This vitelline layer is surmounted by another layer called a jelly coat. The genital products are conveyed to the cavity of the right renal organ and are freed into the sea through the renal aperture whereby the ova sink and the spermatozoa swim (Bevelander 1988).

1.4 METHODS OF INVESTIGATION OF ABALONE TISSUES

Histology is defined as the study of the fine structure of animal and plant tissues, the aim of which is to obtain thin sections of these tissues in as life-like a manner as possible (Bucke 1989). Histological staining is the standard technique used in shellfish research. It is applied to the study of both normal and diseased organisms but requires extensive knowledge and experience (Bower 2003). Histology involves the fixation, processing and sectioning of tissues. Staining techniques commonly employed for light microscopy include standard histological and histochemical methods, immunohistochemical techniques and enzyme histochemical techniques.

For histological analysis specimens must be preserved/fixed and embedded in paraffin wax or some other support medium. A fixative is a compound that maintains tissues and prevents tissue decay and autolysis. For marine molluscs and crustaceans, Davidson's

fixative is the recommended fixative (Bucke 1989). Tissue processing aims to embed the tissue in a solid medium firm enough to support the tissue and give it sufficient rigidity to enable thin sections to be cut. As most fixatives are water based and wax is not miscible with water, it is necessary to process the tissue so that it can be impregnated with wax (Anderson and Bancroft 2002). Paraffin wax is the most popular embedding medium for histology as it is cheap and can be handled with ease. Microtomy is used to section the embedded tissue block for microscopic examination. Some proteins and enzymes are labile and are damaged by the steps of fixation and processing. This damage is avoided by means of tissue freezing and sectioning in a cryostat (Anderson and Bancroft 2002). Liquid nitrogen is used to freeze the tissue initially to -196°C and this procedure eliminates the need for dehydrating and clearing agents.

1.4.1 Immunohistochemistry

Immunohistochemical techniques have allowed for the specific localisation of antigen molecules in tissues and cells with improved specificity of staining reactions and the identification of a wider range of cell and tissue components, compared to classical dye staining methods that identify a limited number of proteins, enzymes and tissue structures (DeMey and Moeremans 1986). It is a technique for identifying cellular or tissue constituents through antibody-antigen interactions.

Antigenic proteins have one or more antibody binding sites. These specific topographical regions are comprised of a small number of amino acids and are known as epitopes (Miller 2002). Antibodies belong to a class of serum proteins called immunoglobulins and are produced as part of an individual's response to foreign

antigens (DeMey and Moeremans 1986). The amino acid side chains of the variable domain of the antibody form a cavity that is geometrically and chemically complementary to a single type of antigenic epitope (Miller 2002). The antigens and antibodies are held together by hydrogen bonds, electrostatic forces and van der Waal's forces. Five types of antibody arise in the immune system of higher vertebrates: IgA, IgD, IgE, IgG and IgM. Monoclonal antibodies are specific to one epitope of the antigen molecule while polyclonal antibodies are comprised of many antibodies that are reactive with a variety of epitopes on the antigen molecule (Miller 2002).

There are two methods of immunohistochemistry that are typically employed. The *Direct Method* uses a labelled primary antibody and is a short and simple method but lacking adequate sensitivity. The *Indirect Method* is more commonly utilised because it is a rapid, inexpensive and sensitive process. The primary antibody binds to the antigen in the tissue section and a second tracer-conjugated antibody is applied to the section that binds to the primary antibody. The complex that forms is then visualised by the application of a chromogenic substrate (Miller 2002). Immunohistochemistry is very sensitive and specific as a result of specialised techniques such as the Avidin Biotin Complex Method (ABC) method (Fig. 1.5).

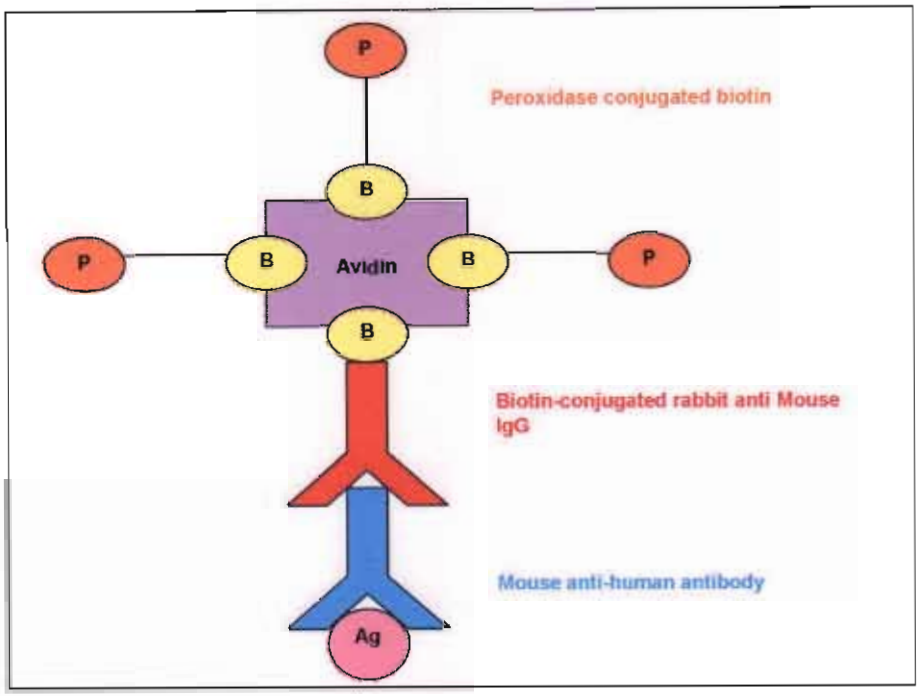

Figure 1.5. The Avidin Biotin Complex (Adapted From: Miller, K. D. 2002. *Immunocytochemical Techniques*. In: Bancroft, J. D. and Gamble, M. (eds.), Theory and Practice of Histological Techniques. (5th ed.) Churchill Livingstone; Edinburgh. pp.421-465).

As previously stated, some antigens can be damaged or destroyed by fixatives. To prevent this frozen sections can be employed, however they are not always available so routinely processed samples have to undergo antigen retrieval which enhances immunostaining by retrieving antigens masked by aldehyde cross-linking of proteins during fixation (Shi et al. 2001). Antigen retrieval techniques include proteolytic enzyme digestion and/or heat induced methods using various buffers. Immunohistochemistry is a valuable method for cell phenotyping and is employed worldwide for cell and tumour analysis. Labels can be attached which facilitate antigen detection by light, fluorescence or electron microscopy.

1.4.2 Western Blotting

Immunohistochemical staining is often reinforced by protein purification techniques such as immunoblotting and western blotting (Fig. 1.6). Immunohistochemistry and protein blotting share the same fundamental principles, as both techniques identify specific proteins using antibodies (Reece 2004). One major difference between these two methods is protein extraction. With blotting, proteins are initially extracted from tissues using a lysis buffer. The proteins are separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) which is the most widely used method for the analysis of protein samples that separates proteins according to size (Baines 2001). The proteins are electrophoretically transferred from the gel onto a membrane (nitrocellulose or polyvinylidene difluoride (PVDF)) using a transfer buffer. As the buffer flows through the gel onto the membrane the proteins move with the buffer and become trapped on the membrane. The membrane is stained with primary and secondary antibodies and the target proteins are visualised using a chemiluminescence detection system or chromogenic substrates such as diaminobenzidine-peroxide (DAB) or horseradish peroxidase (HRP). If a band is visualised at the correct molecular weight on the membrane then the protein has been successfully extracted and detected indicating its presence within the tissue (Fig. 1.6).

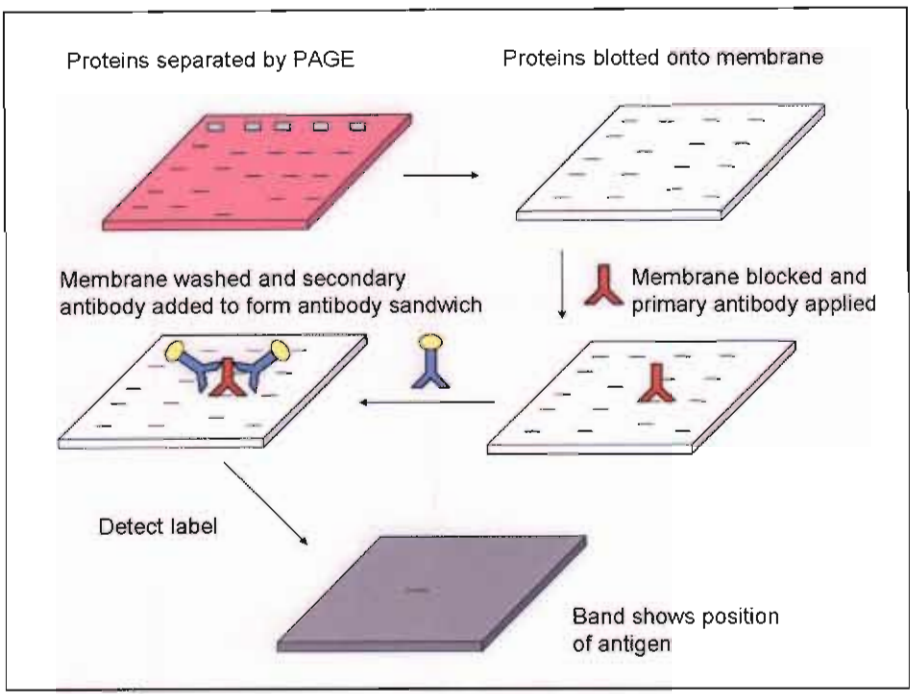

Figure 1.6. Western Blotting (Adapted From: Reece, R. J. 2004. Analysis of Genes and Genomes. John Wiley & Sons Ltd.: West Sussex. p. 104).

1.4.3 Histochemistry

There are numerous methods that can be employed in the visualisation and quantification of different kinds of substances and cells present within tissues. Routine histological and histochemical methods are used to stain cells and tissues, while more specialised histochemical methods are needed for staining particular cells and components that are indicative of molecular changes occurring within the cells (Stoward 1983). Histochemistry describes any technique in which a chemical reaction is involved in colouring a tissue, whether it is staining with dyes or not (Horobin 2002). Histochemistry generates invaluable information about the various constituents of cells and tissues within living organisms and can be used universally in many scientific disciplines. There are countless types of chemical substances present in varying

proportions in cells, such as nucleic acids, carbohydrates, lipids, proteins, peptides, enzymes, hormones and inorganic salts or metals.

Histochemical Demonstration of Enzymes

Enzymes are proteins and must be active to be visualised, which requires careful time preservation by freezing and the presence of a chromogenic substrate (Culling 1963). Enzyme histochemistry can identify cellular components which yields information about cell function and metabolic activity (Drury and Wallington 1980). Enzyme histochemistry allows for the demonstration of enzyme activity by providing co-factors or substrates on which the enzyme can act to produce a coloured deposit or reaction with additional chemical agents or chromogens. They are usually metal ions such as manganese or magnesium (activators) or compounds such as nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) which are co-enzymes. Enzymes are classified into groups according to their effect on substrates (Bancroft 2002).

Histochemical Demonstration of Carbohydrates

In the histochemical demonstration of carbohydrates the two main entities to be considered are mucins or mucosubstances and polysaccharides. Glycogens are polysaccharides that are widely distributed in animal tissues (Drury and Wallington 1980). The role of glucose as a precursor for glycogen synthesis is well understood while the function of mucins in tissues has yet to be made explicit (Totty 2002). Carbohydrates have been long been visualised in paraffin tissues using variations of the PAS technique which uses Schiff's reagent for the demonstration of aldehydes produced after hydrolysis with hydrochloric acid (Culling 1963). Other standard techniques

include the basic dye methods such as alcian blue (Steedman 1950), carmine (Best 1906) and aldehyde fuchsin (Gomori 1950). Mucins can be subdivided into different types such as neutral and acid mucins. Neutral mucins are carbohydrates that are made up of acetylated hexose units (Drury and Wallington 1980). Acid mucins include weakly or strongly sulphated mucins, carboxylated mucins and sulphated sialomucins (Totty 2002). Other carbohydrates that can be histochemically demonstrated include chitin, starch and cellulose.

Histochemical Demonstration of Lipids

Lipids can be defined as any one of a group of fats characterised by their insolubility in water and describe any naturally occurring fats and fat-like substances (Culling 1963; Jones 2002). Lipids are classified according to their chemical structure and include true fats such as esters of fatty acids and glycerol, lipids such as phospholipids, cerebrosides and waxes, sterols such as cholesterol and ergosterol and hydrocarbons such as squalene and carotene. Lipids usually arise in the form of droplets or bound to other tissue entities. Free lipid droplets are destroyed upon exposure to alcohols, xylene and paraffin wax which eliminates the possibility of paraffin processing (Jones 2002). Frozen tissues are best used to preserve and demonstrate lipids in tissues.

Histochemical Demonstration of Pigments and Minerals

Pigments are defined as substances occurring in living matter that absorb visible light and will differ in origin, chemical constitution and biological significance (Churukian 2002). Pigments are organic or inorganic compounds that remain insoluble in most solvents. They are classified as endogenous, exogenous or artefact pigments. Endogenous pigments are substances that are produced within the cells and tissues of an

organism and usually serve a physiological function or are by-products of normal metabolic processes. Exogenous pigments are acquired from the external environment by an organism either by inhalation or implantation in the skin. Most exogenous pigments are minerals. Artefact pigments are deposits of artefactually produced material caused by interactions between tissue components and chemicals (Churukian 2002).

1.5 PATHOGENS OF ABALONE

Global demand for abalone has significantly increased in recent years, however wild stocks of abalone have declined in number resulting from overexploitation as well as the spread of infectious disease (Bower 2003; Gozlan et al. 2006). Shellfish aquaculture facilities have increased in number to compensate for this decline in wild populations. The potential of infectious disease to cause high mortalities within shellfish populations has led to increased awareness of transferable diseases along with increased attempts to detect pathogenic organisms. In 1992, Landau suggested that the feature that makes abalone culture attractive to prospective farmers is that these animals usually have no significant disease or parasite problems, though cases of bacterial and protozoan infections have been reported (Lester and Davis 1981; O'Donoghue et al. 1991; Liu et al. 1995; Nicolas et al. 2002). Prior to 2000 only six severe pathogens/diseases (*Vibrio fluvialis*, *Labyrinthuloides haliotidis*, *Perkinsus olseni*, sabellid polychaete, withering foot syndrome and amyotrophia) that caused mortality in abalone were reported in the available literature (Bower 2000). In 2003, Bower published an updated paper on emerging abalone diseases, indicating that other infectious diseases have also been detected and added to the list of all known pathogens (12) which includes both serious pathogens and pathogens of lesser concern. Therefore, in the last decade or so the

number of abalone diseases has in fact doubled, indicating the ever pressing need for the development of rapid diagnostic techniques.

In the following section six diseases/pathogens that are now considered to be the most severe causes of mortality amongst abalone shellfish populations will be described. They include: *Vibrio* spp., *Perkinsus* spp., *Candidatus Xenohaliotis californiensis* causing withering syndrome, bacteria causing withering syndrome, *Labyrinthuloides* spp., *Haplsoporidia* spp. and an unknown pathogen causing amyotrophia.

1.5.1 *Vibrio* Species

Vibrio is a Gram negative, rod shaped bacterium that causes blister disease or vibriosis in abalone shellfish (Bower 2000). *Vibrio* causes systemic infections of the soft tissues of larvae leading to tissue necrosis and death (Bower et al. 1994). Blisters form on the foot of the adult shellfish and the resulting inflammatory response causes the denaturation of connective tissues and muscle fibres (Bower et al. 1994). Abalone is susceptible to many different species of *Vibrio*: *V. harveyi; V. splendidus II; V. carchariae; V. fluvialis II* and *V. parahaemolyticus*. Different strains of *Vibrio* have been encountered in many species of abalone throughout the world. *Vibrio fluvialis II* caused 50-60% mortality among wild and cultured *Haliotis discus hannai* in Dalian, China (Liu et al. 1995). Disease outbreaks caused by *V. harveyi* and *V. splendidus I* occurred among cultured *Haliotis rubra* and *Haliotis laevigata* in Tasmania, Australia (Bower 2003). *Vibrio carchariae* was isolated in Kanagawa Prefecture, Japan, where it caused mass mortalities among *Haliotis diversicolor supertexta* populations (Nishimori et al. 1998). The same species of *Vibrio* was also suspected as the cause of mass mortalities among *H. tuberculata* along the coast of Brittany and Normandy in France

(Nicolas et al. 2002). Outbreaks of mass mortality among cultured small abalone *Haliotis diversicolor supertexta* Lischke occurred in Taiwan in 1998 (Lee et al. 2001). The causative agents were identified as *V. alginolyticus* and *V. carchariae*. More recently two new *Vibrio* species, *Vibrio neonatus* and *Vibrio ezurae* were isolated from the gut of Japanese abalone (Sawabe et al. 2004) and *Vibrio parahaemolyticus* has lately caused mass mortalities in *Haliotis diversicolor supertexta* Lischke in China (Cai et al. 2006).

1.5.2 *Perkinsus* Species

Perkinsus is a protistan parasite that causes severe problems in abalone in various parts of the world (Bower 2000). *Perkinsus olseni* affects abalone but other species such as *P. atlanticus, P. chesapeaki, P. andrewsi, P. marinus, P. mediterraneus* and *P. qugwadi* have been identified in other shellfish species (Elston et al. 2004). To date only *P. olseni* and *P. marinus* are considered to cause disease that is notifiable to the OIE (Office International des Épizooties) (OIE 2003). The species of abalone that have been recorded as being susceptible to Perkinsosis are: *H. cyclobates, H. laevigata, H. rubra,* and *H. tuberculata*. It is not known if other species of abalone can contract this parasite. *Perkinsus* is a protistan parasite of the genus *Perkinsus* (OIE 2003). Perkinsosis affects a wide variety of molluscs on all five continents, with dramatic consequences in many areas, causing severe economic losses (Villalba et al. 2004). Proliferation of the parasite causes disruption of the connective tissue and epithelial cells of the tissues. Yellow brown abscesses usually form in the flesh of the abalone that measure 8 mm or more in diameter and contain a creamy-brown deposit. In heavy infections, these abscesses occur throughout the foot and mantle (Goggin and Lester 1995). In Australia *Perkinsus* has severely affected both *Haliotis laevigata* and *Haliotis rubra* (Goggin and Lester

1995; Jones and Creeper 2006). In the 1970s, *Perkinsus* was identified in the flesh of blacklip abalone (*H. rubra*) while in the 1980s *H. laevigata* almost disappeared from the shore of the Gulf of St. Vincent, South Australia due to heavy infections of this protozoan (Goggin and Lester 1995). There were two subsequent outbreaks of *Perkinsus* in *H. laevigata* in the 1990s. Between 1986 and 1992 *Perkinsus* was identified, not only in abalone but also in scallops, oysters, ark shells and cockles in Australia (Goggin and Lester 1995). *Perkinsus* has been identified as the causative agent of disease in clams from Chesapeake Bay, Maryland (Dungan et al. 2002), along the Pacific coast of North and Central America (Elston et al. 2004) and in oysters along the Gulf of Mexico to Long Island Sound, United States (Sunila et al. 2001). Epizootic mortalities amongst Manila clams along the west and south coasts of Korea were caused by *Perkinsus* spp. (Park and Choi 2001).

1.5.3 *Candidatus Xenohaliotis californiensis* causing Withering Syndrome

The aetiological agent of withering syndrome a rickettsiales-like organism and has been placed in the Family Rickettsiaciae. Friedman et al. (2000) identified the bacterium as a unique taxon and proposed the provisional status of '*Candidatus Xenohaliotis californiensis*'. This Gram negative, obligate intracellular bacterium is found within membrane bound vacuoles in the cytoplasm of abalone gastrointestinal cells as well as in enzyme secreting cells of the digestive gland (Gardner et al. 1995). Withering syndrome is an epizootic, fatal, wasting disease of wild and cultured abalone (Friedman et al. 2000). *Haliotis cracherodii*, *Haliotis rufescens*, *Haliotis fulgens* and *Haliotis corrugata* are host to the pathogen causing this disease and a similar disease was reported in *Haliotis discus hannai* in China. Nutrient loss through poor gut absorption as well as the loss of enzyme production results in lethargy, retraction of the visceral

tissues and atrophy of the foot muscle (Gardner et al. 1995). Death usually results within one month of the appearance of clinical signs of the disease and elevated temperatures appear to aid in disease progression (Bower 2000).

There have been reports of withering syndrome from southern California, Baja California, Mexico, and a Rickettsia-like prokaryote was detected in the digestive gland of *H. midae* from South Africa (Bower 2003). Mass mortalities of *Haliotis cracherodii* were reported from the Californian Channel Islands in 1986 and in 1992, 99% of this abalone species was lost to withering disease (Bower 2000). Since the early nineties the disease has migrated northwards along the Californian coast posing a threat to healthy populations of black abalone. In 2001, Huang et al. reported *Vibrio parahaemolyticus* as one of the causative agents of withering syndrome in the small abalone *Haliotis diversicolor supertexta.* Abalone were injected with *V. parahaemolyticus* bacterial cells and withering syndrome was resultant, the same bacterial strain was re-isolated from moribund animals indicating the role of this *Vibrio* strain as a causative agent of the disease.

1.5.4 *Labyrinthuloides* Species

Labyrinthuloides is a protistan parasite or thraustochytrid belonging to the subkingdom Labyrinthomorpha but there are prevailing problems with regards to its classification (Bower 2000). It is a pathogenic parasite of small juvenile abalone and has only been recognised in abalone of less than 0.5 cm shell length (Bower 2000). To date *Labyrinthuloides* has been recorded in both *Haliotis kamtschatkana* and *Haliotis rufescens*. This parasite causes 100% mortality in juvenile abalone destroying the muscle and nervous tissue of the head and foot (Bower 1987). Moribund abalone are

found weakly attached to the substrate and the foot and head tissues of infected shellfish appear swollen due to the overwhelming number of protists within the tissues. This parasite is relatively large and can be detected using a compound light microscope (Bower 2003).

Labyrinthuloides was first detected in the 1980s in an abalone culture facility in British Colombia where the northern abalone, *Haliotis kamtschatkana*, was being cultured for the first time. Within the first two weeks of detection of the parasite, over 90% of small abalone were infected and high mortality rates ensued (Bower 2003). The natural distribution of this parasite is unknown but its extreme versatility means that it can survive on virtually nothing for considerable lengths of time (Bower 2000).

1.5.5 *Haplosporidia* Species

In 2000, an unknown protozoan parasite caused mortalities among juvenile cultured abalone (*Haliotis iris*) (Diggles et al. 2002; Hine et al. 2002). It was subsequently identified as a haplosporidian by studying it at the ultrastructural level using TEM (Transmission Electron Microscopy) (Hine et al. 2002) and ISH (*In situ* hybridisation) techniques (Diggles et al. 2002). Symptoms of the disease were lethargy, weak adhesion to substrata, oedema and pale lesions in the foot and mantle. *Haplosporidia nelsoni* and *H. costale* are a major threat to oysters in California and Japan (Friedman 1996) but the haplosporidian reported in abalone seems to be of a different genus, however, its true identity is unknown to date. *Haplosporidium montforti* was recently identified in *Haliotis tuberculata* but is phylogenetically remote from the haplosporidian of Australian *Haliotis iris* (Azevedo et al. 2006).

1.5.6 Unknown Pathogen causing Amyotrophia

Amyotrophia is a fatal wasting disease caused by an unknown etiological agent but it seems to resemble a virus due to the presence of viral particles located near the nerve trunk of diseased abalone as identified by electron microscopy (Bower et al. 1994). This pathogen causes muscle atrophy in the foot and mantle of juvenile abalone, which ultimately impedes feeding and adhesion to the substrate. Infected abalone have many tumours near the nerve trunk of pedal ganglia and their associated transverse commissures (Nakatsugawa et al. 1999). Mass mortalities have been observed in Japan since the 1980s, in populations of *Haliotis discus hannai* and *Haliotis discus discus* as a result of infection with this unidentified pathogen (Nakatsugawa et al. 1999).

1.6 NUCLEIC ACID-BASED METHODS FOR PATHOGEN DETECTION

Marine invertebrate pathology is a young field of research hence the pressing need for research efforts within this area in relation to the control of marine invertebrate infectious disease. In molluscs, viral, bacterial, rickettsial, chlamydial, protozoan and metazoan pathogens have been described and involved in dramatic mortalities (Mialhe et al. 1995). Current techniques applicable to molluscan disease agents are limited and most of the investigations are based on histological and ultrastructural examinations (OIE 2003). The variety of molluscan pathogens is an indication of the need for strong specialisation in the identification and characterisation of these pathogens. The improvement of pathogen characterisation techniques will lead to the development of new diagnostic methods as alternatives to light microscopy, which is often time-consuming and not extremely well adapted for microorganisms (Mialhe et al. 1995).

Despite the drawbacks of being a slow, insensitive and not very specific method of pathogen detection, histology still remains the foremost reference diagnostic technique. Serological methods cannot be used for diagnostic purposes, as shellfish do not produce antibodies. Immunoassays using monoclonal antibodies or nucleic acid probes can be used for direct detection of certain pathogens and recent efforts to overcome diagnostic problems has led to the development of immunoassays and DNA-based methods that could be incorporated into aquaculture research (Bostock 2002; OIE 2003).

Developments in nucleic acid technology have revolutionised biological research in general. Great advances have also been made in the study of shellfish diseases in the last ten years using DNA based technology. However, these new techniques have not been fully incorporated into fish health legislation (Cunningham 2002). Molecular techniques offer great advantages over microscopic examinations such as specificity, rapidity and sensitivity. More recent polymerase chain reaction-based methods also allow for the rapid quantitation of specific pathogens.

1.6.1 Polymerase Chain Reaction (PCR)

PCR is a well-established technique that allows for the repeated copying of a DNA molecule (Brown 2002). Developed by Kary Mullis in 1983, this nucleic acid technology allows for the direct detection of the pathogen by targeting the genetic material of the organism (Cunningham 2002). It uses PCR primers that target fragments for amplification from a wide range of genes and it allows for the analysis of very small quantities of DNA (Sweijd et al. 2000). The presence or absence of a PCR product indicates if a sample is infected or uninfected by a particular pathogen (Cunningham 2002).

PCR uses *Taq* (*Thermus aquaticus*) DNA polymerase to catalyse the exponential amplification of a DNA fragment from a longer template such as the whole chromosome. *Taq* is isolated from bacteria that grow in thermal vents in the ocean or hot springs. The DNA fragment is defined by two short oligonucleotides (primers) that are complementary to the opposing DNA strands of the template being amplified. Primer design is based upon the sequences of the DNA that is desired to be analysed (Dale and Von Schantz 2002). There are many specialised forms of PCR. Multiplex PCR uses two or more sets of primers in the same reaction, so that more than one sequence can be detected simultaneously from one DNA sample (Lee et al. 2003; Rodkhum et al. 2006).

Quantitative real-time PCR is a highly sensitive technique enabling the amplification and the quantification of a specific nucleic acid sequence (Boyle et al. 2004; Mackay 2004; Vadopalas et al. 2006). Quantification is achieved by monitoring the accumulation of product during the reaction (Dale and Von Schantz 2002; Reece 2004; Takahashi et al. 2005). PCR products can be detected using fluorescent dyes that bind to double stranded DNA or fluorescently labelled sequence-specific probes that are specifically designed to hybridise to the target sequence on the amplified DNA fragment (Vadopalas et al. 2006). One of the most popular fluorescent dyes used in quantitative real-time PCR is SYBR Green I, as it is inexpensive and easy to use (Giglio et al. 2003). SYBR Green I dye is a double stranded DNA binding dye that binds to the minor groove of double stranded DNA during the annealing and elongation phases of the PCR reaction. Fluorescence is emitted when the dye binds to double stranded DNA and can be measured during each cycle, so that the amount of fluorescence is proportional to the amount of PCR product (Dale and Von Schantz 2002; Reece 2004; Miller et al. 2006).

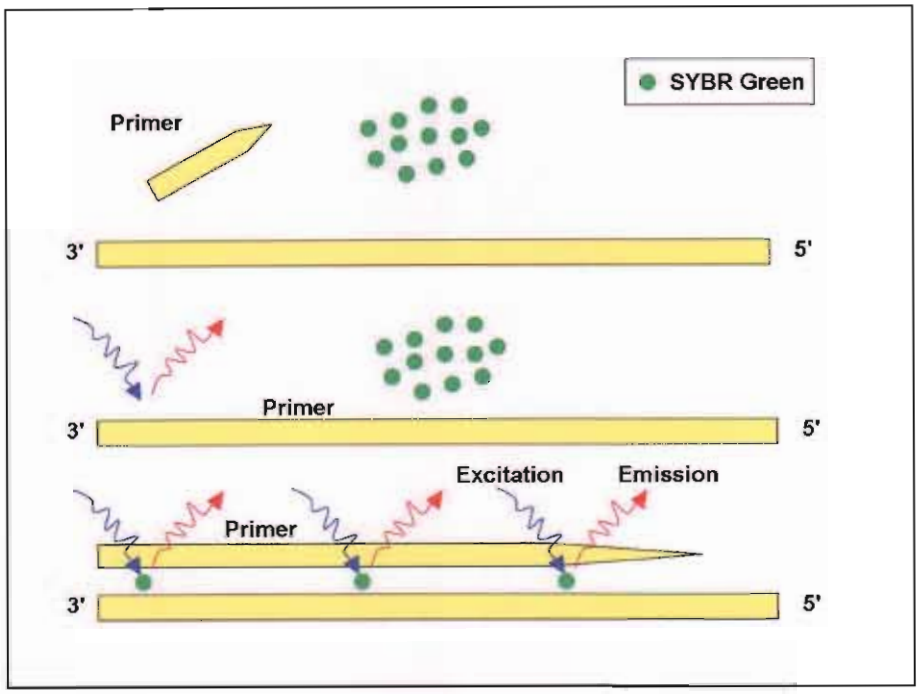

Figure 1.7. Principle of SYBR Green I based detection of PCR products in real-time PCR (Adapted From: www.qiagen.com).

Melt-curve analysis is an important feature of the *LightCycler*® System as it allows for the sequence confirmation of amplified real-time PCR products (Giglio et al. 2003). The melt temperature is specific to each double stranded DNA product and is defined as the temperature at which 50% of the DNA is single stranded and 50% remains double stranded. The melt temperature (T_m) is specific to each PCR product and also allows for the differentiation of specific PCR product from non-specific primer-dimers (Giglio et al. 2003; Miller et al. 2006).

The use of DNA-based techniques offers many advantages over protein-based methods, the main advantage being the stability of DNA. However DNA is also subject to degradation which can be a limitation in PCR methods but this problem can be overcome by targeting small DNA fragments usually below 500 bp in length (Lorente et al. 1997).

1.7 CURRENT METHODS FOR PATHOGEN DETECTION

1.7.1 *Vibrio* Species

The accepted diagnostic techniques currently employed in the detection of *Vibrio*, include histology, electron microscopy and cell culture (Bower 2000). Standard histological protocols give an indication of tissue necrosis and highlight rod-shaped bacteria within the tissues. The standard histological stain used for screening is haematoxylin and eosin which would give an indication of general tissue necrosis but a Gram Twort stain would be more specific for the identification of Gram negative bacteria in paraffin processed tissues. Electron microscopy is also used to identify the bacteria at the ultrastructural level but is not commonly employed in diagnostic laboratories as it is an expensive technique. *Vibrio* colonies can be cultured and isolated from infected abalone using special media, such as marine agar for marine strains or TCBS (Thiosulphate Citrate Bile Salts) agar, which is a general *Vibrio* agar, to identify the source of infection (Bower 2000).

Molecular techniques have been established and are constantly being developed and applied in the detection of *Vibrio* spp. Prokaryotic rRNA (ribosomal RNA) genes contain highly conserved sequences, which allow for the identification and amplification of these rRNA genes. These genes have been used in a PCR assay for *Vibrio anguillarum* (Cunningham 2002). Ribotyping is another technique using rRNA genes that reveals genetic variations to allow for the identification of species or strains (Austin et al. 1997). A multiplex PCR amplification of targeted gene segments followed by DNA-DNA sandwich hybridisation was optimised to detect pathogenic bacteria in shellfish which included three species of *Vibrio*: *V. vulnificus, V. parahaemolyticus* and *V. cholerae* (Lee et al. 2003). A PCR assay was also developed to detect *Vibrio harveyi*

pure bacterial isolates (Oakey et al. 2003). A method of indirect immunofluorescence microscopy was developed for the rapid detection of *V. parahaemolyticus* in oysters (Chen and Chang 1996). In 1992, a research study conducted by Chen et al. developed monoclonal antibodies against *Vibrio* species that infect humans, fish and shellfish. It was discovered that genus-specific monoclonal antibodies were useful for rapid identification of *Vibrios* in acute infections while species-specific monoclonal antibodies were useful for completing the diagnosis (Chen et al. 1992).

Despite the increase in methods for rapid diagnosis of *Vibrio*, there are currently no methods of control once the pathogens have been identified. *Vibrio* bacteria are ubiquitous hence, eradication of this aetiologic agent is impossible. To avoid the introduction of *Vibrio* into other aquaculture facilities or natural stocks, only shellfish known to be disease free should be transplanted.

1.7.2 *Perkinsus* Species

Detection of *Perkinsus* is usually by means of histology and culture. Histological investigations use standard haematoxylin and eosin staining to identify protists in tissue sections. The examination of tissues incubated in Ray's Fluid Thioglycollate Medium (RFTM) is more sensitive than direct observation of paraffin embedded host tissues under the light microscope. Tissues are incubated in RFTM for 4-7 days and tissues are subsequently stained with Lugol's iodine and observed under a light microscope. However, neither technique is specific for *Perkinsus olseni* as both techniques will detect all *Perkinsus* species (Bower et al. 1994). Other diagnostic methods include TEM and PCR. The ribosomal RNA locus of *P. atlanticus* has been characterised using a PCR-based diagnostic assay (Robledo et al. 2000).

A multiplex PCR has been developed for *P. marinus* but has not yet been validated (Penna et al. 2001). Parasite taxonomic verification has been made using a genus-*Perkinsus* SSUrRNA (small subunit ribosomal RNA) gene-specific DNA probe for *in situ* hybridisation on histological samples and its use for parasite identification has been validated (Elston et al. 2004). Direct antibody (polyclonal) fluorescent techniques have been used in *Perkinsus* diagnosis, however these immunoassays detect a range of *Perkinsus* species and are not species-specific (Goggin and Lester 1995). There are no known methods of prevention or control for this disease but it is thought that elevated temperatures may predispose abalone to the disease. As a precautionary method, only abalone free of infection should be imported into areas with no record of *Perkinsus* infection (Bower 2000).

1.7.3 *Candidatus Xenohaliotis californiensis* causing Withering Syndrome

Detection of this pathogen is usually by light microscopy (LM), electron microscopy, squash preparations, ISH and PCR (Friedman et al. 2000). Histology is more sensitive than tissue imprints or squash preparations, however definitive diagnosis may require the use of molecular tools, as this bacterium may be morphologically similar to other marine rickettsial bacteria (OIE 2003). A PCR reaction for the detection of genomic DNA of a Rickettsiales-like prokaryote associated with Withering Syndrome in black abalone has been developed (Andree et al. 2000). ISH is the preferred diagnostic technique as it allows for the visualisation of a specific probe hybridised to the target organism. Antonio et al. (2000) developed *in situ* hybridisation for the detection of Rickettsiales-like prokaryotes. There are no control measures in place for this pathogen once it is detected but reducing temperatures below 15°C in culture facilities could help

reduce the severity of the disease, as it tends to arise in the summer and autumn months (Bower 2000).

1.7.4 *Labyrinthuloides* Species

Diagnostic techniques for *Labyrinthuloides haliotidis* include gross observation of infected organisms, squash preparations that identify numerous spherical protozoa and standard histological techniques that identify the parasite in the tissues of the head and foot. Culturing of *Labyrinthuloides* parasites in minimum essential medium (MEM) and immunoassays have been used in the detection of this parasite (Bower et al. 1989). *Labyrinthuloides* can be destroyed by exposure to a solution of 25 mg of sodium hypochlorite per litre of seawater or treating seawater with 0.97 mg ozone per litre for 25 minutes, this kills many of the zoospores (Bower et al. 1994).

1.7.5 *Haplosporidia* Species

Histology is the foremost technique used in the diagnosis of *Haplosporidia* spp. which allows for the identification of uni-nucleate and multi-nucleate plasmodia in the connective tissue of abalone organs (Bower et al. 1994; Bower 2003). TEM and ISH techniques have also been used in the detection of this pathogen (Diggles et al. 2002; Hine et al. 2002). There are no known methods of prevention or control for this pathogen.

1.7.6 Unknown Pathogen causing Amyotrophia

The methods employed for the detection of amyotrophia include electron microscopy to visualise the viral particles and culture, however isolates from primary cultures did not reproduce the disease (Nakatsugawa et al. 1999). Further research is necessary to

identify the causative agent of amyotrophia and to design methods of control (Bower 2000).

1.7.7 Conclusion on Current Detection Systems

It can be seen from the literature that the application of molecular techniques in marine pathology is very sparse despite their unparalleled advantages. The OIE Manual of Diagnostic Tests for Aquatic Animals has incorporated some molecular methods into its protocols for disease diagnosis but reference laboratories have been slow to adopt these methods (Cunningham 2002). Being a novel area of research, both literature and expertise are scarce and molecular biology is expensive. All of these factors may be contributing to the reticence of some laboratories to adopt these methods (Cunningham 2002). Hence there is a need for increased research efforts in developing molecular diagnostics in the marine arena.

1.8 AIMS OF THIS STUDY

The aims of this study will be to carry out a histological characterisation of two species of abalone shellfish using both immunohistochemical and histochemical techniques, to study its biology, and develop molecular methods for the investigation of shellfish pathogens. This work can be divided into three main components: immunohistochemistry, histochemistry and microbial pathology.

In chapter 2, immunohistochemistry will be used to examine the distribution of cells, structural and functional proteins in abalone tissues. Immunohistochemical techniques will be employed to demonstrate phenotypic antigen expression in abalone shellfish using an array of antibodies that cross react with proteins in a range of different species.

Some proteins are highly conserved throughout evolution while others are only present in closely related species. Currently there are no molluscan antibodies commercially available. Antibodies that react to human and veterinary antigens will be chosen for evaluation on shellfish tissues to determine the extent of cross reaction between species and also to identify the types of cell markers within shellfish tissues.

In chapter 3 abalone histology will be characterised using histochemistry and enzyme histochemistry. There are relatively few studies on the histology and histochemistry of the visceral organs of abalone and the information is fragmented with respect to these two species of abalone. This study will look at the distribution pattern of different types of enzymes, carbohydrates, lipids, pigments and minerals in the specialised organs of abalone.

In chapter 4, molecular methods will be developed for the detection of two prominent pathogens of abalone. A PCR assay will be developed to detect *Vibrio harveyi* (Oakey et al. 2003). A novel PCR assay will be developed to detect *V. harveyi* in alcohol-fixed tissues and paraffin embedded tissues of *Haliotis tuberculata*. A multiplex PCR method will also be developed to simultaneously amplify a 16S rRNA gene of *V. harveyi* and a housekeeping actin gene in abalone. A quantitative SYBR Green real-time PCR method will be developed to detect *V. harveyi* and also to enumerate bacterial load in samples.

In addition a PCR method will be developed to detect DNA extracted from *Perkinsus olseni*. This PCR will be applied to detect part of the ITS region of *P. olseni* in paraffin embedded clam tissues (*Ruditapes decussatus*). This PCR will be developed into a SYBR Green real-time PCR for the detection and quantitation of this parasite. These

nucleic acid-based techniques will be developed to increase the speed of disease diagnosis in aquaculture facilities which could potentially reduce the loss of shellfish stocks to infectious diseases.

CHAPTER 2

CHARACTERISATION OF CELL TYPES IN ABALONE (*HALIOTIS* SPECIES) TISSUES USING IMMUNOHISTOCHEMICAL TECHNIQUES.

2.1 INTRODUCTION

2.1.1 Immunohistochemistry and its Applications

Immunohistochemistry has been extremely influential in the practice of human diagnostic pathology over the past two decades. The recognition of specific epitopes in paraffin embedded tissues using antibodies and enzyme labels is a routine procedure in most diagnostic pathology laboratories (Miller 2002). The labelling of proteins in tissues yields a vast amount of information about the cellular and molecular interactions occurring within the cell. Immunohistochemistry is the technique used by most scientists to identify cell markers, as it remains one of the most effective methods of characterising specific phenotypic antigenic expression and can be applied to invertebrates (Lyons-Alcantara et al. 1999; Lyons-Alcantara et al. 2002).

Immunohistochemistry was developed in the early 20th century and it is a technique concerned with methods based upon the precepts of immunology (Kiernan 1999; Miller 2002). It allows for the demonstration of antigens in tissue sections by the use of specific immunological (antibody-antigen) interactions culminating in the attachment of a visible marker (usually an enzyme) to the antigen (Avrameas and Uriel 1966; Nakane and Pierce 1966). Cell markers are proteins characteristic of certain cell types. They may arise as surface cell markers that are molecules or proteins characteristic of the plasma membrane of a cell or as intracellular proteins that occur within the cell. Cell types and tissues express cell specific markers, which can be identified through immunohistochemical labelling. Immunohistochemistry is an effective tool in studying cell distribution, differentiation and function and can be applied to the study of immune cells and cellular biomarkers such as structural, functional, cell cycle and apoptosis related epitopes.

Vertebrate animals defend themselves against harmful antigens through the secretion of antibodies that are produced in response to foreign antigens. The interaction of the antibody and antigen commonly results in the neutralisation of the toxicity of the antigen (Kiernan 1999; Miller 2002). Antigen-antibody reactions are used throughout the sciences in immunoassays, immunoprecipitation, western blotting and immunostaining.

2.1.2 The Molluscan Immune Response

Invertebrates do not possess immunoglobulins or a powerful acquired immunity as do vertebrates and much less is known about their immune defence mechanisms (Roch 1999). However the invertebrata pre-date the vertebrates by millions of years, so their survival somehow implies that they possess an extremely efficient immune system (Roch 1999).

According to Roch (1999) invertebrates possess an innate, non-adaptive immune system employing a large variety of circulating molecules. These circulating molecules have a role in phagocytic, inflammatory and cytotoxic responses which boost the number of immune reactions available for the defence of these invertebrates. Hemocytes represent the first line of defence in molluscs and are categorised into granulocytes, agranulocytes and hyalinocytes (Bachère et al. 1995; Xue and Renault 2001; Sun et al. 2006). The development of monoclonal antibody technology in the study of molluscan immune cells has led to the development of specific markers for hemocyte sub-population identification (Yoshino and Granath 1983; Xue and Renault 2001; Sun et al. 2006). Previous studies have only used immunohistochemistry to study the hemocytes/immune cells of molluscs, which uses antibodies produced specifically to detect these cells,

allowing for the definition of molluscan hemocyte type and the determination of hemocyte distribution within tissues (Yoshino and Granath 1983; Ottaviani 1989; Noël et al. 1994; Xue and Renault 2001).

2.1.3 Immunodiagnostics in Molluscan Studies

The characterisation of cell types in higher vertebrates has been researched extensively but the information available for invertebrates remains scarce (Baccetti et al. 1984; Lyons-Alcantara et al. 1999). The human antibody repertoire can collectively target thousands of antigens, but so far researchers have produced a very limited range of antibodies to invertebrate antigens (Lyons-Alcantara et al. 2002). Owing to this limitation immunohistochemical techniques have rarely been employed in the study of shellfish.

Infectious disease has emerged as the major constraint to shellfish aquaculture and efforts have focused on disease prevention through the study of pathogen detection and the immune defence mechanisms of shellfish. Therefore, the use of monoclonal antibodies and immunodiagnostic techniques has been concentrated in the study of marine invertebrate immunology and infectious disease (Yoshino and Granath 1983; Mialhe et al. 1988; Noël et al. 1994; Coll and Dominguez-Juncal 1995; Roch 1999; Xue and Renault 2001).

Much work has been done in relation to the characterisation of fish cell types. Coll and Dominguez-Juncal (1995) reviewed the application of monoclonal antibodies in aquaculture with particular emphasis on the immune response of teleost fish. Lin and Dickerson (1992) identified immunogens of molecular weight 48 and 60 kDa in the

protozoan *Ichthyophthirius multifiliis* which play a role in protective immunity of fish to this parasite and Espelid et al. (1987) found that 95% of *Vibrio*-infected salmon antibodies bound to the outer surface antigen of *V. salmonicida*. While many basic studies still need to be carried out in lower vertebrate immunology, Coll and Dominguez-Juncal (1995) have emphasised that further development of monoclonal antibodies and the characterisation of antigenic determinants involved in protection, such as those carried out by Lin and Dickerson (1992) and Espelid et al. (1987) will have new applications in diagnosis and vaccine development for fish. Studies such as these have potential for use in shellfish research.

Immunodiagnostic techniques for the early detection of pathogens in aquaculture are continuously under development using molecular probes and antibodies (Roch 1999). Monoclonal antibodies in particular, are being used to characterise the causative agents of molluscan disease such as protozoa and *Vibrio* bacteria (Mialhe et al. 1988; Boulo et al. 1989; Cochennec et al. 1992). However these studies concentrated on the use of techniques such as immunofluorescent assays (IFA) and enzyme linked immunosorbent assays (ELISA).

Noël et al. (1994) performed immunohistochemical studies on the hemocytes of the mussel *Mytilus edulis* to characterise subpopulations of hemocytes using monoclonal antibodies specific for epitopes in basophilic and eosinophilic granulocytes and indicated their possible use in studying the alteration of determinants in neoplastic hemocytes due to cell transformation. Xue and Renault (2001) incorporated both western blotting and immunohistochemistry into their study characterising the hemocytes of the European flat oyster, *Ostrea edulis*. They identified three groups of

hemocytes: granulocytes, large hyalinocytes and small agranulocytes and reported that oyster granulocytes and hyalinocytes share a common antigen. They also compared the distribution patterns of granulocytes in adult and developing animals noticing an absence of granulocytes in early larval stages, which indicate an immature immune system in oyster larvae. Their results suggested that monoclonal antibodies are valuable tools in the study of invertebrate hemocyte development. Sun et al. (2006) more recently investigated the morphology, structure, function and classification of hemocytes in the oyster *Crassostrea ariakensis*. While immunohistochemical techniques are utilised within shellfish research, they are primarily used in the study of disease and in gaining a better understanding of the defence mechanisms of molluscs. In the study presented here immunohistochemistry will be used to characterise both structural and proliferative proteins in abalone tissues which have not been characterised before. This may facilitate the development of abalone cell lines through the identification, isolation and culture of target cells, which could prove useful in studies of disease and environmental toxicity.

2.1.4 Immunohistochemistry and Cell Evaluation in Molluscs

In vertebrates and invertebrates, primary cell culture methods have paved the way for understanding the cellular and molecular events occurring during cell proliferation, cell differentiation and cell metabolism (Lebel et al. 1996). Proteins in invertebrates have been studied to a certain extent. In invertebrates substances homologous to vertebrate growth factors have been identified through immunocytochemical and biochemical analyses. These insulin-like growth factors have been localised in the digestive system and the brain of different molluscs and are both functionally and structurally similar to mammalian insulin like peptides (ILPs) (Ebberink et al. 1989). Their involvement in

body and shell growth and energy metabolism in molluscs has been proposed by Scvala et al. (1993). Epidermal growth factor (EGF) is another vertebrate growth factor that has been localised in many invertebrates such as the nematode *Caenorhabditis elegans*, the mussel *Mytilus edulis*, the common fruit fly *Drosophila melanogaster* and many species of sea urchin (Greenwald 1985; Livneh et al. 1985; Hursh et al. 1987; Yang et al. 1989; Rutlegde et al. 1992; Inoue et al. 1995).

Franchini et al. (1996) used immunohistochemistry to examine the presence of a group of peptides derived from milk proteins or caseins in the tissues of lower vertebrates and invertebrates. They reported that the casein fragments as recognised by the antibodies and their biological properties, have been preserved throughout evolution. The majority of vertebrate peptides can be traced back through evolution but they may have evolved for similar or different purposes (Franchini et al 1996). Furthermore Scharrer (1990) described neuropeptides as molecules that are widely distributed throughout the animal kingdom and appear to have arisen before the development of a primitive nervous system. Various neuropeptides have been reported in marine molluscs including schistosomin, an anti-gonadotropic neuropeptide in the marine snail *Conus spurius* and the RF-amide (Arg-Phe amide) neuropeptides in the pond snail *Lymnaea stagnalis* and the gastropods *Buccinum undatum* and *Busycon canaliculatum* (Hordijk et al. 1991; Maillo et al. 2002; Moulis 2006). FMRF-amide neurotransmitters have been identified in the abalone *Haliotis asinina* (Panasophonkul et al. 2004).

The histology of prosobranch and abalone shellfish has been studied (Bevelander 1988; Voltzow 1994) but the information available on these shellfish remains incomplete. Immunohistochemistry has also been used in the detection of cytokeratins in fish and

invertebrates (Markl and Franke 1988; Markl et al. 1989; Bunton 1993; Diogo et al. 1994; Karabinos et al. 1998). Cells of the digestive gland of the Norwegian prawn, *Nephrops norvegicus* and the shrimp *Pandalus borealis* were characterised using immunohistochemistry (Lyons-Alcantara et al. 1999; Lyons-Alcantara et al. 2002) and the localisation of the nuclear protein PCNA (Proliferating Cell Nuclear Antigen) within the nuclei of both fish and prawns was also established using this technique (Suzuka et al. 1989; Ortego et al. 1994).

More recently, immunohistochemical methods using rabbit and mouse polyclonal antibodies, were employed to locate serotonergic and FMRF-amidergic (Phe-Met-Arg-Phe-amide) neurons within the cerebral, pleuropedal and visceral ganglia of *Haliotis asinina*, highlighting the importance of these two neurotransmitters in the physiologic and behavioural responses of this animal (Panasophonkul et al. 2004). Immunohistochemistry was also used to demonstrate that GABA (gamma-aminobutyric acid) a major molluscan neurotransmitter, is present in high concentrations in the sensory epithelial cells of *Haliotis asinina* Linnaeus (Wanichanon et al. 2004). It has been observed that abalone larvae show an absolute requirement for GABA, for the induction of genetically programmed behavioural and developmental metamorphosis to the juvenile form, hence its use as a settlement inducer in aquaculture (Morse et al. 1979a; Morse et al. 1980).

Intermediate filaments are vital cytoskeletal structural components of differentiated cells and are divided into separate classes depending on tissue type whether it is epithelial, neural, connective tissue or glial cells. There are five classes of intermediate filaments: keratins, vimentin, desmin, neurofilament (NF) and glial fibrillary acidic

protein (GFAP) (Barr et al. 2002). It has been suggested that all vertebrate and invertebrate intermediate filament proteins share a common antigenic determinant (Pruss et al. 1981). Proteins homologous to vertebrate cytoplasmic intermediate filament proteins (type I and II proteins include keratins and type III proteins include homopolymeric proteins such as vimentin and desmin) were identified in the invertebrate *Branchiostoma lanceolatum* (Karabinos et al. 1998). Cytokeratins have been well researched in fish and crustaceans (Markl and Franke 1988; Markl et al. 1989; Lyons-Alcantara et al. 1999; Lyons-Alcantara et al. 2002). Many structural proteins are highly conserved in evolution and can be detected through the cross reaction of antibodies to human antigens with epitopes in shellfish tissues.

Structural proteins such as intermediate filament proteins, proliferation proteins and neural proteins have been investigated to some degree in fish and invertebrates (Markl and Franke 1988; Karabinos et al. 1998; Lyons-Alcantara et al. 1999). More recently specific neural markers have been studied in abalone shellfish (Panasophonkul et al. 2004; Wanichanon et al. 2004). Structural and proliferative proteins have not been studied in these molluscs owing to the lack of shellfish antibodies to these proteins. This research will focus on the immunohistochemical evaluation of cell types such as structural and functional proteins in shellfish tissues that have not been previously characterised. In this study a wide range of antibodies to fish and human antigens will be investigated to determine their cross reactivity with similar proteins present in abalone tissues using immunohistochemistry. Where positive immunohistochemical results are observed, western blotting will be employed to confirm the presence of these specific proteins in abalone tissues.

2.2 MATERIALS AND METHODS

2.2.1 Specimen Collection

- On December 8th 2002, 10 abalone shellfish (3 *Haliotis discus hannai* of 5 cm shell length, 4 *H. discus hannai* of 2 cm shell length and 3 *Haliotis tuberculata* of 5 cm shell length) were collected from the Boet Mór shellfish farm, Clifden, Co. Galway, Ireland.
- On January 21st 2004, 14 abalone shellfish (7 *H. discus hannai* of 5 cm shell length and 7 *H. tuberculata* of 5 cm shell length) were collected from the Boet Mór shellfish farm, Clifden, Co. Galway, Ireland.
- On November 9th 2004, 33 abalone shellfish (5 *H. discus hannai* of 5 cm shell length, 8 *H. discus hannai* of 2 cm shell length, 10 *H. tuberculata* of 5 cm shell length and 10 *H. tuberculata* of 2 cm shell length) were collected from the Boet Mór shellfish farm, Clifden, Co. Galway, Ireland

Abalone of 5 cm shell length were approximately 3 years old (adult) and abalone of 2 cm shell length were approximately 1 year old (young). Male and female abalone were collected. Specimens were transported to Dublin in a container of seawater through which air was bubbled. All shellfish appeared healthy upon arrival in Dublin, were immediately placed in 50% alcohol: 50% seawater and prepared for experimental work (paraffin processing or protein extraction).

2.2.2 Sample Preparation for Western Blotting

Shellfish used for protein extraction were placed in 50% alcohol and 50% seawater for 30 min. Samples were immediately dissected and a small piece of each organ was embedded in OCT (Optimal Cutting Temperature) compound and snap frozen in liquid

nitrogen (-196°C). The frozen blocks were wrapped in aluminium foil and stored at -70°C.

2.2.3 Sample Preparation for Immunohistochemistry

After 30 min in absolute alcohol, the molluscs were removed from their shells and placed in Davidson's fixative (Appendix A) for 24 h. An incision was made lengthwise down the foot muscle of each mollusc to allow penetration of the fixative. Individual organs (digestive gland, foot, gill, gonad, hypobranchial gland, mantle and renal organs) were dissected from shellfish of 5 cm in length and animals less than 2 cm in length were cross-sectioned longitudinally.

2.2.4 Paraffin Processing of Fixed Tissue to Paraffin Wax

All samples preserved in Davidson's fixative were placed on an automated processor (Histokinette) for 18 h and passed through the following solutions: 10% formalin, spirit (95% ETOH), absolute alcohol (x5), xylene (x3) and paraffin wax (x2). Tissues were embedded in paraffin wax and 5 µm sections were cut using a microtome. All sections were set onto adhesive APES (3-aminopropyltriethoxysilane) coated slides and incubated in an oven at 56°C for 2-3 h. Slides were then stained using the ABC immunostaining protocol as follows.

2.2.5 Immunostaining Protocol

Shellfish and human tissues (human tissues known to express specific antigens for selected antibodies were used as positive controls) were stained using Vectastain® Elite Avidin-Biotin Complex kits (Vector). Sections were dewaxed and rehydrated to water before staining. The following antigen retrieval methods were used to enhance immunostaining. (a). Microwaving: dewaxed and rehydrated slides were placed in 500

ml citrate buffer (2.1 g citric acid in 1 litre of distilled water at pH 6 using 2 M NaOH) microwaved (800 W) for 18 min and incubated in hot buffer for a further 20 min. (b). Proteolytic Digestion: 0.1% protease (10 U/mg) (*Streptomyces griseus*, Type 24, Sigma) in PBS (Phosphate Buffered Saline) (Appendix A) was heated to 37°C. Dewaxed slides were incubated for 10 min in enzyme solution. Following pre-treatment sections were treated with 3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity. Following rinsing in water, sections were incubated in PBS for 5 min, dabbed dry and tissue sections were circled with a water repelling Dako Pen (DakoCytomation™ Ireland Ltd.). Sections were covered with normal horse serum (1:100 universal kit; 1:67 mouse kit) for 5 min. They were then drained and the primary antibody diluted in PBS was applied to sections for 60 min (Tables 2.1 and 2.2). The slides were rinsed in PBS (x3) and incubated in biotinylated secondary antibody (1:25 universal kit; 1:50 mouse kit) for 15 min. Slides were washed in 3 changes of PBS and incubated for 15 min in ABC reagent (1:25 with both kits). Sections were rinsed in PBS buffer and the chromogen Diaminobenzidine-peroxide (DAB) (0.06 mg ml^{-1} in PBS with 0.03% hydrogen peroxide) was applied for 5 minutes to develop a brown reaction product. Slides were stained in Mayer's Haematoxylin for 1 min and dehydrated through graded alcohols, cleared in xylene and coverslipped with Distrene Plasticiser Xylene (DPX). Human tissues used as positive controls are listed in Tables 2.1 and 2.2 (Human tissues supplied by other DIT ethically approved projects). For negative controls, all reagents were applied to abalone tissues except the primary antibody. For verification of positive results, staining was performed on samples in triplicate.

All antibodies were tested on two species of both male and female abalone, *Haliotis discus hannai* and *Haliotis tuberculata*. However, the best stained section for each

antibody was only chosen for photography, whether it was observed in *H. discus hannai* or *H. tuberculata*. The same results were obtained for each species and sex unless otherwise stated in the results section.

Table 2.1

Specifications and Controls for Antibodies that react with Antigens in Human/Mammalian Species

Antibody*	Specificity/ Reactivity	Source of Immunogen	Clone	Optimal Dilution	Antigen Retrieval	Positive Control
CD 20	B cells	Human	L26	1:1200	Microwave	Tonsil
CD 34	Endothelial cells/stem cells	Human	QBEnd 10	1:50	Microwave	Tonsil
CD 68	Macrophages	Human	KP1	1:50	Microwave	Tonsil
Chromogranin A	Neuronal/ Endocrine	Human	DAK-AE	1:500	Microwave	Ileum
Cytokeratin AE1/3	Keratins	Human	AE1/3	1:800	Protease	Pancreas
Cytokeratin 8	Keratins	Human	4.1.18	1:50	Microwave and Protease	Pancreas
Ki67	Proliferating cells	Human	MIB-1	1:25	Microwave	Tonsil
Cytokeratin MNF 116	Keratins	Human	MNF 116	1:120	Protease	Ileum
Collagen IV	Basement membrane	Human	CIV22	1:450	Microwave	Kidney
Desmin	Muscle intermediate filament	Human	D 33	1:50	Microwave	Intestine
34βE12	Keratins	Human	34 βE12	1:20	Protease	Pancreas
LP 34	Keratins	Human	LP34	1:50	Protease	Skin
Muscle Specific Actin (MSA)	Skeletal, cardiac and smooth muscle	Human	HHF 35	1:100	Microwave	Pancreas
Neurofilament	Neural intermediate filaments	Porcine	2F 11	1:50	Microwave	Cerebellum
Neuron Specific Enolase (NSE)	γγ-enolase enzyme	Human	BB/NC/ VI - H14	1:600	Microwave	Intestine
Proliferating Cell Nuclear Antigen (PCNA)	Proliferating cells	Rat	PC10	1:600	Microwave	Tonsil
S100	Glial cells of CNS/PNS	Bovine	S100	1:400	Microwave	Skin/Sal gland
Synaptohysin	Neuronal synapses	Bovine	SY38	1:20	Microwave	Pancreas
Vimentin	Muscle cells	Bovine	VIM 3B4	1:50	Microwave	Tonsil
Von Willebrand Factor (VWF)	Endothelial cells	Human	F8/86	1:75	Microwave	Tonsil

*All antibodies used were supplied by DakoCytomation™ Galway, Ireland.

Table 2.2

Specifications and Controls for Antibodies that react with Antigens in both Vertebrate and Invertebrate Species

Antibody	Specificity/ Reactivity	Source of Immunogen	Clone	Supplier	Optimal Dilution	Antigen Retrieval	Positive Control
Cytokeratin AE1	Keratins	Human	AE1	Serotec	1:100	Protease	Pancreas
Laminin	Basement Membrane	Mouse	Polyclonal	Abcam	1:100	Protease	Liver
Neurofilament	Neural intermediate filaments	Rat	RmdO-20	Zymed	1:50	Microwave	Cerebellum
PCNA	Proliferating cells	Rat	PC10	Serotec	Neat	Microwave	Tonsil
Vimentin	Muscle cells	Porcine	V9	Serotec	1:80	Microwave	Smooth muscle

2.2.6 Cell Culture for Western Blotting

The following cell lines were grown in culture and protein was extracted from fully confluent cells which served as positive controls in western blotting experiments.

- HeLa – Epithelial tumour cell line (cytokeratins and intermediate filaments).
- CHO – (Chinese Hamster Ovary) Epithelial and fibroblast cell line (Vimentin).
- BALB/C – Brain microvascular endothelial cell line (Neuron Specific Enolase).

Cell lines (frozen in 10% DMSO (dimethyl sulfoxide)) in complete RPMI 1640 medium (Appendix A), were removed from liquid nitrogen storage and thawed at room temperature (RT). All cell culture procedures were carried out in sterile fume cupboards in a designated cell culture laboratory. To remove the DMSO, 1 ml of cells was pipetted into 10 ml of complete RPMI 1640 medium. The cells were centrifuged at 2000 rpm for 5 min. The medium was decanted off, cells were resuspended in 25 ml of complete medium, transferred to a large sterile flask and incubated at 37°C for 2 days.

Sub-culturing of cells

The confluency of cells was checked using an inverted microscope and cells were sub-cultured at 80% confluency. 5 ml of trypsin (25 g/l) (Appendix A) was thawed and added to 5 ml of 0.02% Versene (Appendix A) which was then used to harvest and sub-culture the cells. The medium was decanted off the cells which were washed twice in sterile PBS and an equal volume of trypsin/Versene (5 ml:5 ml) was added to cells. The flask was placed back in the incubator at 37°C for 5 minutes to allow cells to round up. When cells had lifted off the surface of the flask they were transferred to a sterile universal container and 5ml of complete medium was added to inactivate trypsin. Cells

were centrifuged at 2000 rpm for 5 min, the medium was poured off and cells were resuspended in 25 ml fresh medium. Cells were transferred to 2 fresh sterile flasks which were incubated at 37°C.

2.2.7 Protein Extraction for Western Blotting

Two different protocols for protein extraction were used to ensure efficient extraction of protein from two different sample types: fresh abalone tissues and cell lines. For protein extraction from abalone tissues, approximately 200 mg of fresh frozen tissue was macerated and suspended in 2 ml SDS (Sodium Dodecyl Sulphate) lysis buffer (Appendix A) and boiled for 3 min (Cummins and Hanna 2004). The sample was homogenised using a pellet pestle motor (Kontes, New Jersey, USA) and then boiled for a further 5 min. Samples were centrifuged at 13,000 rpm for 10 min and the supernatant was collected for storage at – 20°C.

Protein was extracted from cell lines using NP40 Triple detergent lysis buffer (Appendix A). The medium was decanted from a flask of confluent cells which were washed twice with cold PBS. 3 ml of NP40 lysis buffer was added to the flask and cells were scraped off the surface of the flask into the lysis buffer using a sterile cell scraper. The lysis buffer containing the cells was transferred to 1.5 ml eppendorfs and the suspensions were centrifuged at 13,000 rpm for 20 min at 4°C. The supernatant containing the protein was stored at -20°C.

2.2.8 SDS-PAGE of Proteins

Protein expression and purification was confirmed by SDS-PAGE analysis allowing for the visualisation of proteins and an estimation of the molecular weight of the proteins. The plates and gaskets were assembled and clamped for ATTO mini gel electrophoresis.

A 12% resolving gel was prepared (Appendix A). The solution was mixed and poured into the gel template up to 1.5 cm from the top of the notched plate. The gel was overlain with 0.1% SDS and allowed to set for 20 min. When the resolving gel was set, the 0.1% SDS solution was decanted off the resolving gel and the stacking gel (Appendix A) was added to the gel plate. The comb was inserted and the gel was left to set for a further 20 min. The ATTO gel electrophoresis rig was set up and filled with 1X running buffer (Appendix A). 10 µl of protein and 5 µl of 2X SDS loading buffer (Appendix A) were boiled for 5 min and loaded onto the gel. The gel was run at a current of ~60 mA and the voltage was set at ~110V for 1½-2 hours.

2.2.9 Bradford Assay for Estimation of Protein Concentration

Soluble protein concentrations (µg/ml) of abalone tissues samples and human cell lines were analysed using the Bradford protein assay (Bradford 1976). A stock standard solution of Bovine Serum Albumin (BSA) was prepared at a concentration of 1 mg/ml. A top working standard (T.W.S.) of 20 µg/ml was prepared, from which a series of standards in the range 2-25 µg/ml was prepared (Table 2.3). Protein samples were diluted 1/500 with distilled water to bring them within the assay soluble protein concentration range. 0.2 ml of Bradford dye reagent (Biorad) concentrate was added to 1 ml of each standard and protein sample, mixed by vortexing and left to stand at RT for 5 min. All standards and samples were assayed in triplicate. The spectrophotometer was

zeroed with a water blank at 595 nm before the absorbance readings of the standards and samples were taken. A standard curve was prepared by plotting the mean absorbance values against protein concentration (µg/ml) of the series of standards (Table 2.3; Fig. 2.1).

Table 2.3

Absorbance values (595 nm) and Average for each Standard in Triplicate

BSA (ug/ml)	Absorbance 1	Absorbance 2	Absorbance 3	Mean Value
1	0.762	0.753	0.742	**0.752**
2	0.948	0.952	0.922	**0.94**
5	1.188	1.2	1.129	**1.172**
10	1.555	1.553	1.550	**1.553**
15	1.809	1.794	1.818	**1.807**
20	1.895	1.896	1.916	**1.902**

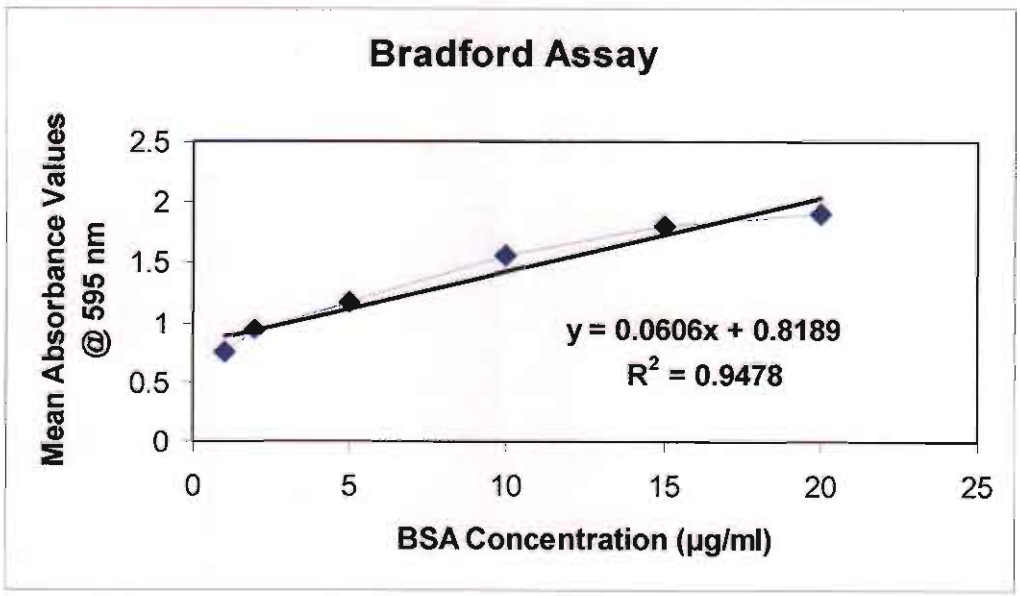

Figure 2.1. Plot of BSA standard curve with BSA concentration (µg/ml) versus absorbance at 595 nm.

From this graph the unknown protein concentration (Table 2.4) of the samples was determined using the formula Y = MX + C, where Y is the absorbance, M is the slope of the line, X is the protein concentration and C is a constant by linear regression.

Table 2.4

Concentration of Protein extracted from Abalone Tissue Samples and Cell Lines

Protein Sample	Protein Concentration (µg/µl)	Concentration of Protein in 10 µl of Sample
Abalone Sample 1	0.63 µg/µl	6.3 µg
Abalone Sample 2	0.7 µg/µl	7 µg
HeLa cell line	1.13 µg/µl	11.3 µg
CHO cell line	0.81 µg/µl	8.1 µg

2.2.10 Western Blotting: Semi-Dry Transfer

The gel plates were separated and the gel was removed. The electrobot apparatus consisted of six pieces of blotting paper (cut exactly to the size of the gel) soaked in transfer buffer (Appendix A). Three pieces of blotting paper were placed on the anode of the western blotter. The PVDF membrane was cut to the exact size of the gel soaked in methanol (to allow opening of the pores in the membrane), then soaked in transfer buffer and placed on top of the blotting paper. The resolving gel was placed on top of the membrane, followed by three pieces of soaked blotting paper. The cathode was placed over the sandwich and the electrodes and power supply connected. The blotter was run at 2 mA per cm^2 of gel for 2 h.

2.2.11 Immunostaining of Protein Blots

The blotting paper was discarded and the membrane was removed and washed briefly with TBS (Tris Buffered Saline) buffer (Appendix A). The membrane was kept moist from this point onwards. The membrane was incubated in 5% BSA blocking solution (Appendix A) overnight at 4°C on a shaker at a low setting to prevent non-specific binding of the primary antibody. After blocking the membrane was washed briefly in TBS and the primary antibody was added to the membrane at the appropriate dilution in 5% BSA blocking solution (Table 2.5). The membrane was incubated in primary antibody at RT for 2 h on a shaker. The membrane was washed in 5% BSA blocking solution (x3) for 15 min. The biotinylated secondary antibody (diluted in 5% BSA blocking solution) was added and the membrane was incubated for 1 h at RT. The membrane was washed in 5% BSA blocking solution (x3) for 15 min. The ABC reagent (diluted in 5% BSA blocking solution) was added and the membrane was incubated for 1 h at RT. The membrane was washed in 5% BSA blocking solution (x3) for 15 min. The membrane was finally incubated in DAB (0.06 mg ml^{-1} in PBS with 0.03% hydrogen peroxide) to produce a brown reaction product for the protein of interest. The membrane was washed in 5% BSA blocking solution and allowed to air dry prior to storage in the dark.

Table 2.5

Specifications and Controls for Antibodies used in Western Blotting

Antibody	Source of Immunogen	Clone	Supplier	Optimal Dilution	Positive Cell line Control	Molecular Weight (kDa)
Cytokeratin AE1	Human	AE1	Serotec	1:100	Hela	40; 48; 50; 56.
Cytokeratin MNF	Human	MNF 116	DAKO	1:120	Hela	45-56.5
PCNA	Rat	PC10	Serotec	Neat	Hela	36
PCNA	Rat	PC10	DAKO	1:600	Hela	36
Vimentin	Porcine	V9	Serotec	1:80	CHO	57

2.2.12 Microscopic Photography

Histological images were captured using two different types of photography. Digital images were captured using a Canon Power Shot S50 digital camera (Canon Inc., Japan), attached to a Leica DM LS2 microscope (Leica, Germany). Images were captured using the Canon Remote Capture computer programme and saved as jpegs. Photographs were also taken using a manual Wild Leitz MP S52 camera and Wild MPS 46 Photoautomat panel, attached to a Leitz Labor Lux S microscope (Leitz, Heerburg, Switzerland). Photographic films were developed, scanned into a computer and images were saved as jpegs.

2.3 RESULTS

2.3.1 Application of Antibodies that bind with Human/Mammalian Antigens to Abalone Tissues

Twenty antibodies that react to mammalian antigens were applied to abalone tissues (Table 2.1). Cross sections of whole animals were used in preliminary trials and any positive staining was subsequently verified by staining individual shellfish organs. Three out of the twenty mammalian antibodies tested reacted with antigens in abalone tissues: PCNA, cytokeratin MNF 116 and NSE. All human positive control tissues were positive for each antibody tested and negative controls were negative (Table 2.1). Images of all positive control tissues can be viewed in Appendix B. A brown reaction product indicates a positive reaction.

2.3.1 (a) Proliferating Cell Nuclear Antigen (PCNA)

Human tonsil was used as a positive control for PCNA (Fig. 2.1.1). In test samples PCNA positive cells were concentrated in the nuclei of the gonadal tubules in the reproductive organs of male abalone and the cell nuclei were strongly positive for the presence of this protein (Fig. 2.1.2). Female abalone tissues were not tested with this PCNA antibody. Epithelial cells of the digestive system were also positive when stained with this antibody (Fig. 2.1.3). However the level of expression in the digestive epithelia was not as strong as in the cells of the gonad. A negative abalone control was used and no staining was observed (Fig. 2.1.4).

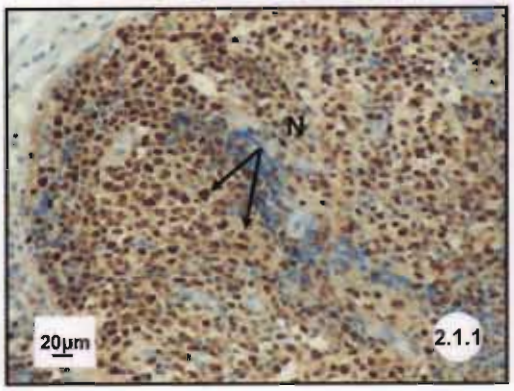

Figure 2.1.1: Immunoperoxidase staining for PCNA in the nuclei (N) of human tonsil (x400 manual camera).

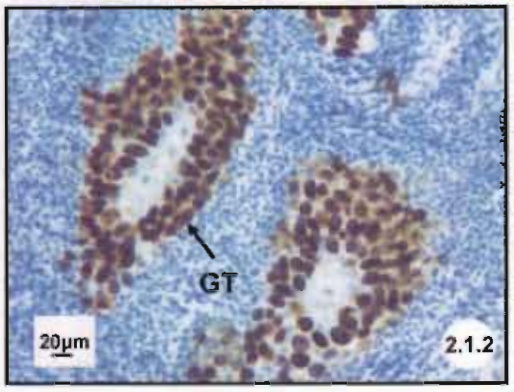

Figure 2.1.2: Immunoperoxidase staining for PCNA in the nuclei of the male gonadal tubules (GT) of *Haliotis discus hannai* (x400 manual camera).

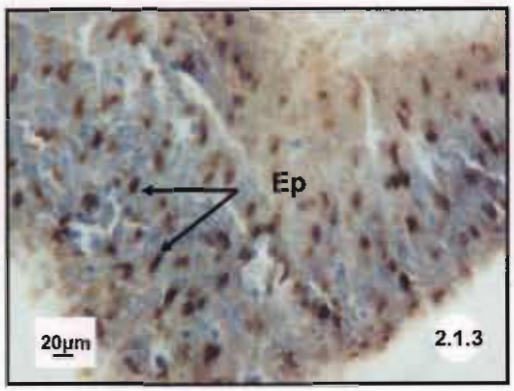

Figure 2.1.3: Immunoperoxidase staining for PCNA in the nuclei of the digestive epithelial cells (Ep) of *Haliotis discus hannai* (x400 manual camera).

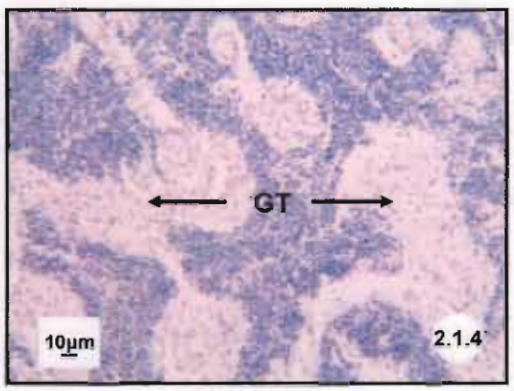

Figure 2.1.4: Negative control for PCNA immunoperoxidase staining in the gonadal tubules (GT) of *Haliotis discus hannai* (x400 digital camera).

2.3.1 (b) Cytokeratin MNF 116 (Keratins 5, 6, 8, 17, 19)

Human intestine was used as a positive control for cytokeratin MNF (Fig. 2.2.1). Expression of cytokeratin MNF was concentrated on the surface of the intestinal epithelial cells of abalone (Fig. 2.2.2). No staining was observed in the negative control slide (Fig. 2.2.3).

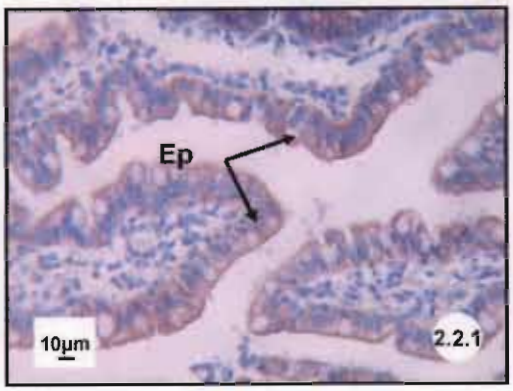

Figure 2.2.1: Immunoperoxidase staining for cytokeratin MNF in the epithelial cells (Ep) of human ileum control tissue (x400 digital camera).

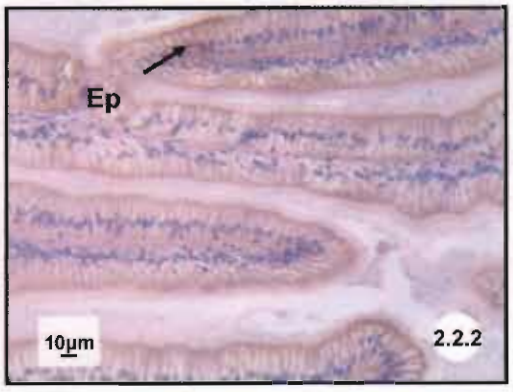

Figure 2.2.2: Immunoperoxidase staining of cytokeratin MNF in epithelial cells (Ep) of the intestine of *Haliotis discus hannai* (x400 digital camera).

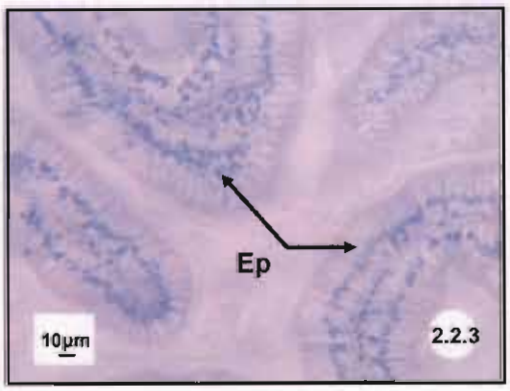

Figure 2.2.3: Negative control for immunoperoxidase staining of cytokeratin MNF in the intestinal epithelial cells (Ep) of *Haliotis discus hannai* (x400 digital camera).

2.3.1 (c) Neuron Specific Enolase (NSE)

The antibody NSE is directed against a neural protein and is found in abundance in nerve cells. Human ileum served as a positive control (Fig. 2.3.1). NSE positive cells were also observed within the connective tissue of the digestive gland of abalone (Fig. 2.3.2). No staining was observed in the negative control slide (Fig. 2.3.3).

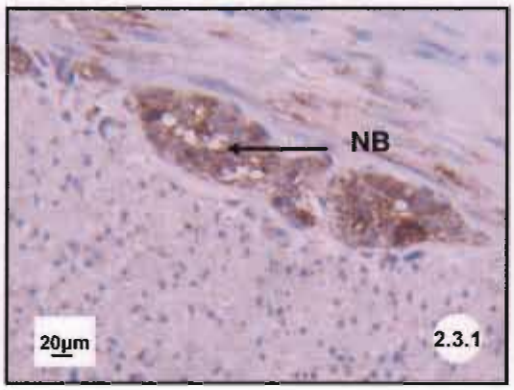

Figure 2.3.1: Immunoperoxidase staining of NSE in neural bundles (NB) in human ileum (x400 manual camera).

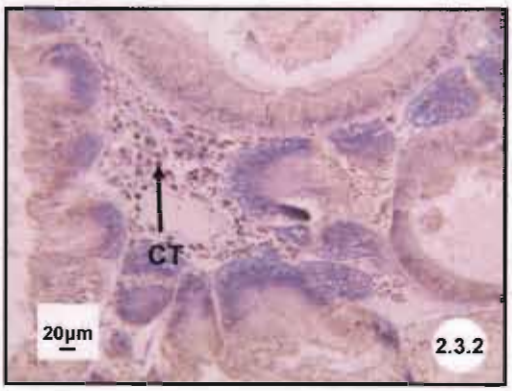

Figure 2.3.2: Immunoperoxidase staining of NSE in the connective tissue (CT) of the digestive gland of *Haliotis discus hannai* (x200 digital camera).

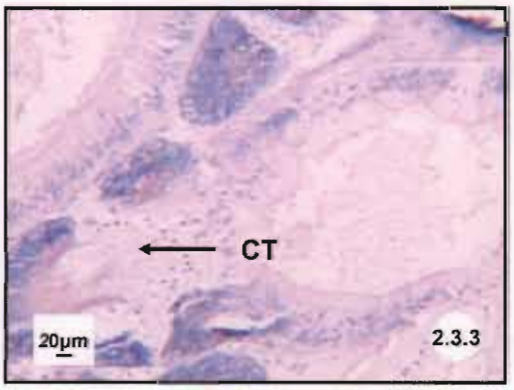

Figure 2.3.3: Negative control for immunoperoxidase staining of NSE in the connective tissue (CT) of the digestive gland of *Haliotis discus hannai* (x200 digital camera).

In this first section of immunohistochemical staining, a panel of 20 antibodies in total was applied to abalone tissues (Table 2.1) and positive results were observed with PCNA, cytokeratin MNF and NSE. The other antibodies tested reacted positively with human keratins, proliferating cells, neural cells, muscle cells and endothelial cells (Appendix B) but they did not react positively with antigens in abalone tissues.

2.3.2. Application of Antibodies that bind with other Vertebrate and Invertebrate Antigens to Abalone Tissues

Five antibodies that bind to both vertebrate and invertebrate antigens including PCNA, cytokeratin AE1, vimentin, laminin and neurofilament were tested on cross sections of abalone tissues and on individual organs such as the digestive gland, epipodium, foot, gonad and kidney (Table 2.2). Four antibodies gave positive results but antigens were not detected in abalone tissues when tested with the neurofilament antibody. All human positive control tissues were positive for each antibody tested and negative controls were uniformly negative (Table 2.2). Images of all positive control tissues can be viewed in Appendix B.

2.3.2 (a) Proliferating Cell Nuclear Antigen (PCNA)

The PCNA antibody (Serotec) used in this section of the study reacts with antigens in both vertebrates and invertebrates. Human tonsil was used as a positive control tissue (Fig. 2.4.1). In abalone tissues both the male and female gonads expressed PCNA. The nuclei of large but immature ova in the female gonad were positive for this protein (Fig. 2.4.2) and negative controls showed no staining (Fig. 2.4.3). The male gonadal tubules displayed the same pattern previously observed with the PCNA antibody (DAKO) to the human antigen (Fig. 2.4.4) and negative staining was observed in the negative control tissue (Fig. 2.4.5).

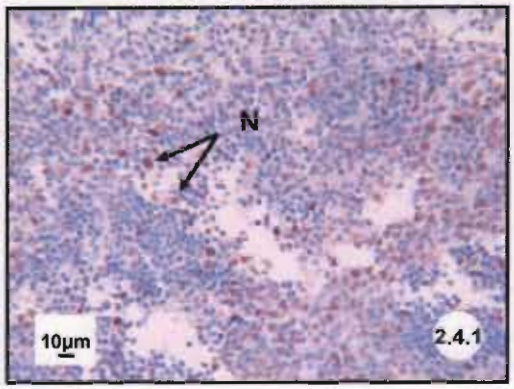

Figure 2.4.1: Immunoperoxidase staining of PCNA in the nuclei (N) of human tonsil (x400 digital camera).

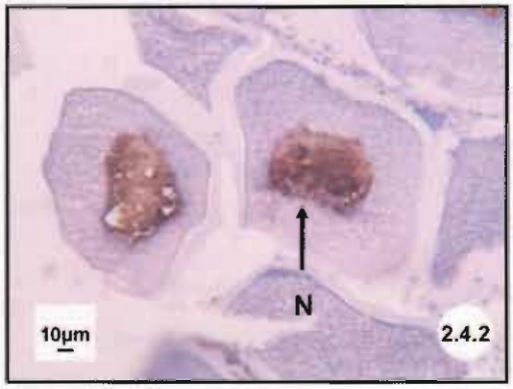

Figure 2.4.2: Immunoperoxidase staining of PCNA in the nuclei (N) of eggs in the gonad of female *Haliotis discus hannai* (x400 digital camera).

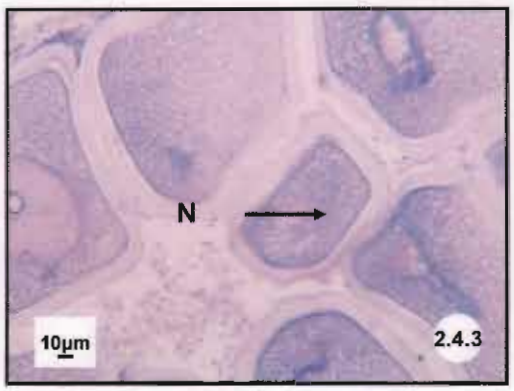

Figure 2.4.3: Negative control for immunoperoxidase staining of PCNA in the nuclei of eggs (N) of female *Haliotis discus hannai* (x400 digital camera).

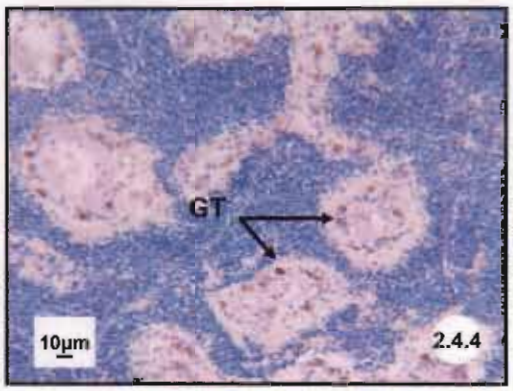

Figure 2.4.4: Immunoperoxidase staining of PCNA in the nuclei of the gonadal tubules (GT) of male *Haliotis discus hannai* (x400 digital camera).

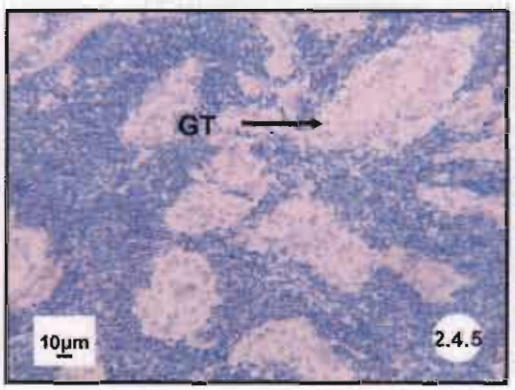

Figure 2.4.5: Negative control for immunoperoxidase staining of PCNA in the nuclei of the gonadal tubules (GT) of male *Haliotis discus hannai* (x400 digital camera).

2.3.2 (b) Cytokeratin AE1 (Keratins 9, 10, 13, 14, 15, 16, 19)

Strong expression of cytokeratin AE1 was observed in the human pancreas positive control (Fig. 2.5.1). Cytokeratin AE1 was expressed in some but not all epithelial cells of the digestive system of abalone tissues. The positive reaction observed was not uniform, rather scattered throughout the cells of the intestine (Fig. 2.5.2). This cytokeratin was also expressed in the pedal epithelia. No staining was observed with the negative control tissue (Fig. 2.5.3).

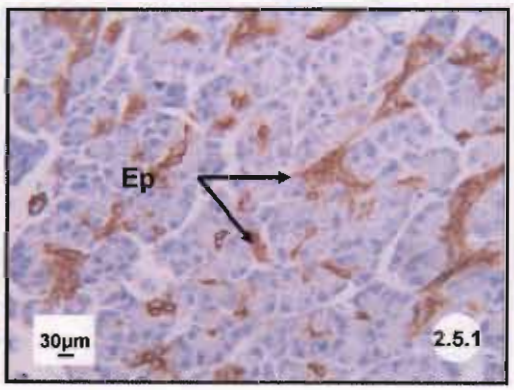

Figure 2.5.1: Immunoperoxidase staining of cytokeratin AE1 in the epithelial cells (Ep) of human pancreas (x200 manual camera).

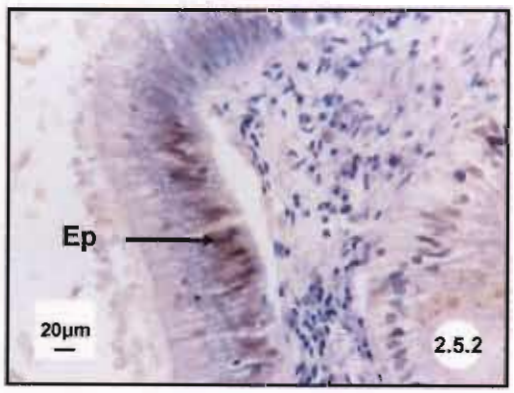

Figure 2.5.2: Immunoperoxidase staining of cytokeratin AE1 in the epithelial cells (Ep) of the intestine of *Haliotis discus hannai* (x400 manual camera).

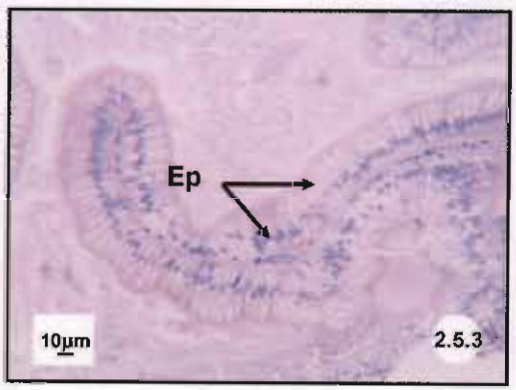

Figure 2.5.3: Negative control for immunoperoxidase staining of cytokeratin AE1 in the intestinal epithelial cells (Ep) of *Haliotis discus hannai* (x400 digital camera).

2.3.2 *(c) Vimentin*

Two different cell clones of vimentin antibody (VIM 3B4 (Table 2.1) and V9 (Table 2.2)) were used in this study but only the V9 clone detected the antigen in abalone tissues. Smooth muscle served as a positive control (Fig. 2.6.1). A granular staining pattern was expressed in the epithelial cells of the right renal organ of *Haliotis tuberculata* only, with this antibody (Fig. 2.6.2) and no staining was observed with the negative control (Fig. 2.6.3).

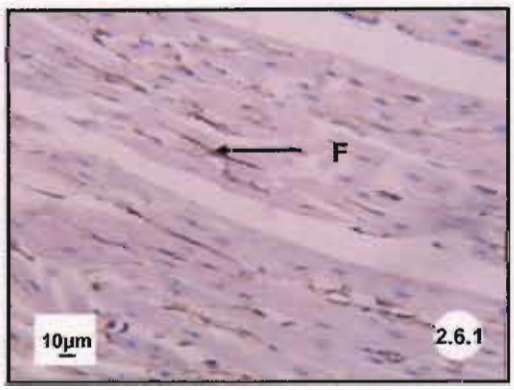

Figure 2.6.1: Immunoperoxidase staining of vimentin (V9) in fibroblasts (F) of human smooth muscle (x400 digital camera).

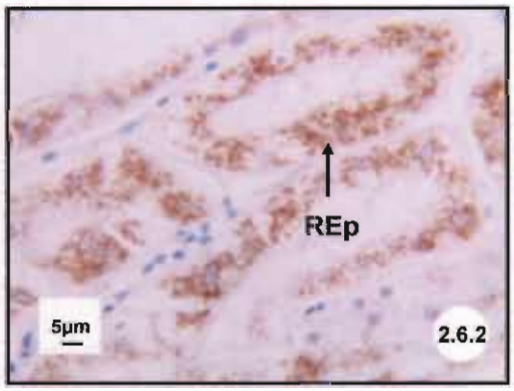

Figure 2.6.2: Immunoperoxidase staining of vimentin (V9) in renal epithelial cells (REp) of *Haliotis tuberculata* (x1000 oil digital camera).

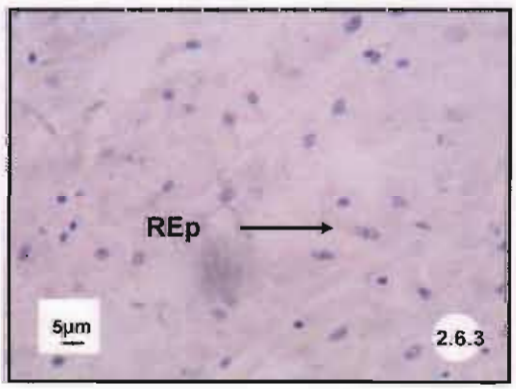

Figure 2.6.3: Negative control for immunoperoxidase staining of vimentin (V9) in the renal epithelial cells (REp) of *Haliotis tuberculata* (x400 digital camera).

2.3.2 *(d) Laminin*

Laminin is an abundant glycoprotein in basement membrane. Human liver was used as a positive control tissue for laminin (Fig. 2.7.1). Laminin was expressed in the basement membrane below the pedal epithelial layer of the abalone foot (Fig. 2.7.2). No staining was observed in the negative control tissue (Fig. 2.7.3).

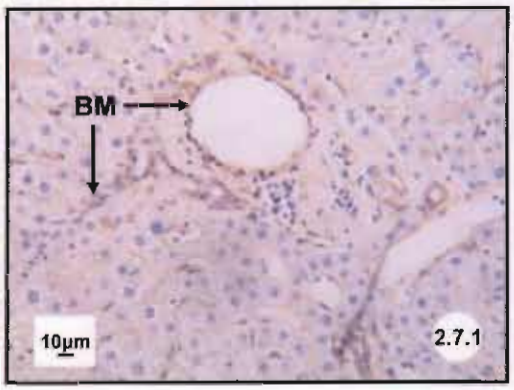

Figure 2.7.1: Immunoperoxidase staining of laminin in the basement membrane (BM) of human liver (x200 digital camera).

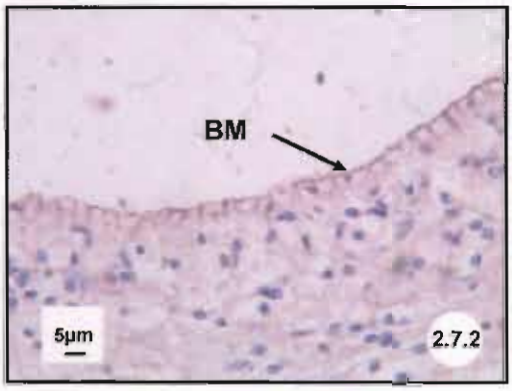

Figure 2.7.2: Immunoperoxidase staining of laminin in the basement membrane (BM) of the foot of *Haliotis discus hannai* (x1000 oil digital camera).

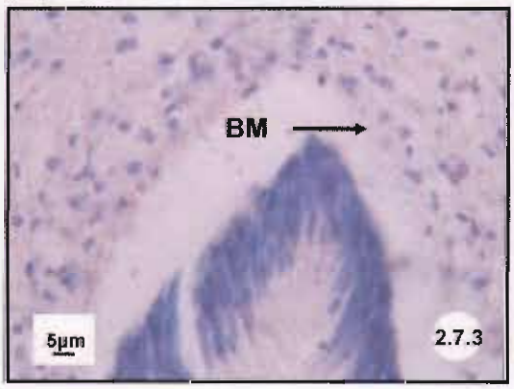

Figure 2.7.3: Negative control for immunoperoxidase staining of laminin in the basement membrane (BM) of *Haliotis discus hannai* (x1000 oil digital camera).

In this second section of immunohistochemical staining, a panel of 5 antibodies in total was applied to abalone tissues (Table 2.2) and positive results were observed with PCNA, cytokeratin AE1, laminin and vimentin. The human positive control stained positively with the neurofilament antibody (Appendix B) but it did not react positively with abalone tissues.

2.3.3 Western Blotting Results

Proteins identified in abalone tissues by immunohistochemistry were further investigated and identified by ABC/DAB detection in western blotting using SDS-PAGE and transfer to PVDF membranes. Western blot analysis was successful using controls for PCNA, cytokeratin MNF, cytokeratin AE1, and vimentin but confirmation of NSE and laminin was unsuccessful. Western blot analysis confirmed the presence of PCNA, cytokeratin MNF, cytokeratin AE1 and vimentin in abalone tissues. Different concentrations of protein were loaded onto the gels for western blot analysis (Table 2.4).

Western blot analysis with both PCNA (DAKO and Serotec) antibodies identified clear bands with a molecular weight of approximately 36 kDa in HeLa positive controls and whole protein extracted from abalone tissues (Fig. 2.8.1, Fig. 2.8.2). Higher concentrations of protein explain the stronger bands observed with HeLa positive controls compared with abalone protein. In Fig. 2.8.2 specific bands between 83 and 175 kDa can be observed in *H. discus hannai* despite a lower concentration of protein than the positive control.

Western blotting also confirmed the presence of cytokeratins in abalone using MNF and AE1 antibodies. The broad spectrum antibody cytokeratin MNF which detects a wide range of proteins, revealed proteins with molecular weights of between 25 and 175 kDa in the HeLa positive control (Fig. 2.8.3). With abalone samples, 3 strong bands with molecular weights of 100, 83 and 70 kDa approximately were identified and weaker bands were observed in the 32.5-47.5 kDa range (Fig. 2.8.3). A very strong band at 83 kDa can be observed in the protein extract of *H. tuberculata* in Fig. 2.8.3. The lower

molecular weight bands correspond to keratins 5, 6, 8, 17 and 19 while the high molecular weight proteins identified do not correspond to known cytokeratins.

The antibody cytokeratin AE1 identifies proteins with molecular weights of 40-56.5 kDa. A single band with a molecular weight of 56.5 kDa approximately was identified in both the positive HeLa control cells and abalone samples, which corresponds to cytokeratin 10 (Fig. 2.8.4).

Protein bands were detected in abalone with the vimentin antibody using western blotting (Fig. 2.8.5). A band was observed in the CHO positive control which corresponds to the 57 kDa band expected with vimentin. In abalone samples weak bands were observed in the 47.5 kDa-62 kDa range. A very strong band and two weaker bands were identified with molecular weights of between 83-175 kDa in the *H. tuberculata* sample. The strong band observed in Fig. 2.8.5 is very specific despite lower concentrations of abalone compared to the CHO positive control.

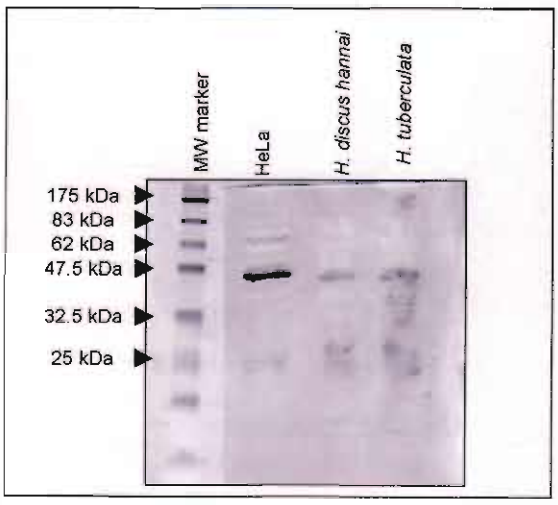

Figure 2.8.1: Western blot of HeLA cells (11.3µg) and abalone tissue extracts (*H. discus hannai* and *H. tuberculata*) (6.3 µg; 7 µg) using PCNA (DAKO).

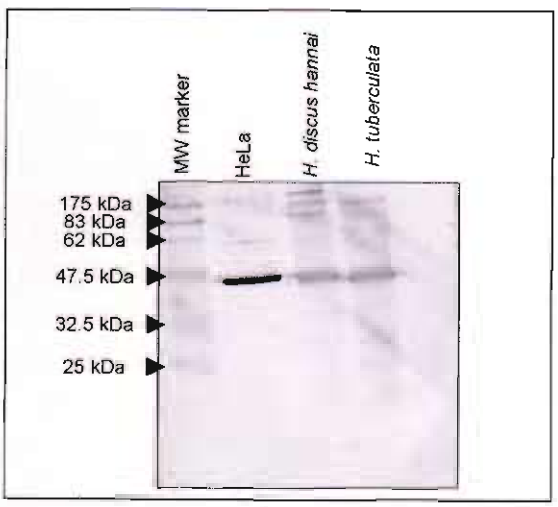

Figure 2.8.2: Western blot analysis of HeLA cells (11.3 µg) and abalone tissue extracts (*H. discus hannai* and *H. tuberculata*) (6.3 µg; 7 µg) using PCNA (Serotec).

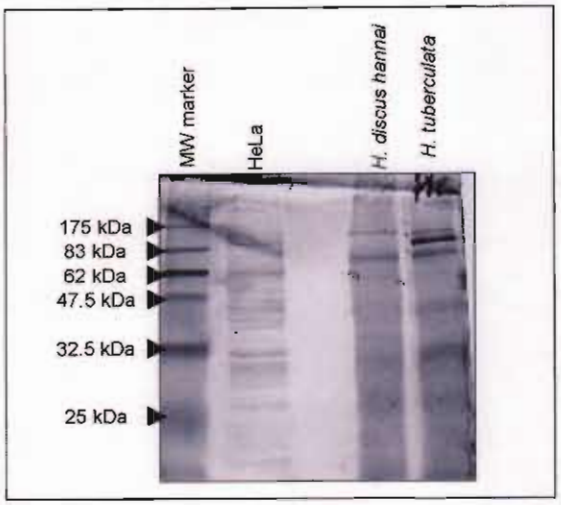

Figure 2.8.3: Western blot of HeLA cells (11.3 µg) and abalone tissue extracts (*H. discus hannai* and *H. tuberculata*) (6.3 µg; 7 µg) using cytokeratin MNF 116 (DAKO).

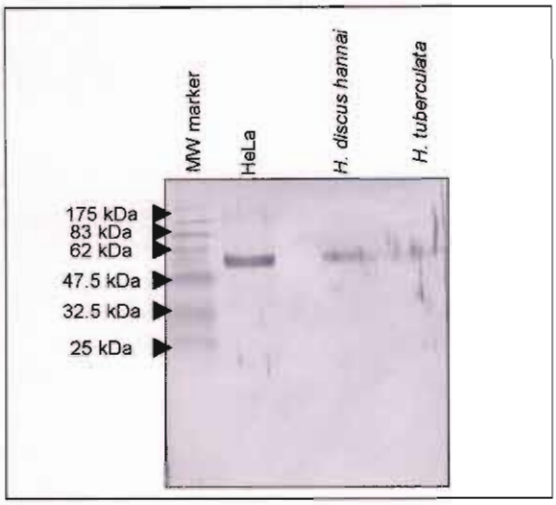

Figure 2.8.4: Western blot of HeLa cells (11.3 µg) and abalone tissue extracts (*H. discus hannai* and *H. tuberculata*) (6.3 µg; 7 µg) using cytokeratin AE1 (Serotec).

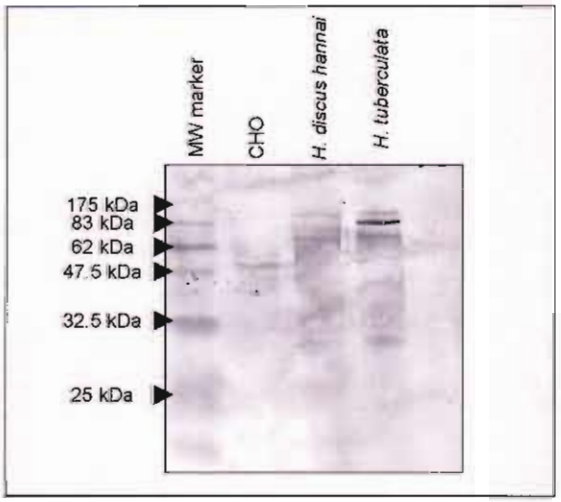

Figure 2.8.5: Western blot of CHO cells (8.1 µg) and abalone tissue extracts (*H. discus hannai* and *H. tuberculata*) (6.3 µg; 7 µg) using vimentin V9 (Serotec).

2.4 DISCUSSION

Immunohistochemistry is a highly valued technique that allows for the study of functional and structural entities within tissues. It allows for the analysis of protein distribution within tissues and has potential for application in marine research. However, the lack of specific antibodies to shellfish antigens remains a major limitation. In an attempt to overcome the unavailability of shellfish specific antibodies this study investigated twenty five antibodies known to react with a wide range of both vertebrate and invertebrate proteins. The results of this study indicate the evolutionary conservation of PCNA, cytokeratins, vimentin, NSE and laminin.

2.4.1 Proliferating Cell Nuclear Antigen (PCNA)

PCNA is a multifunctional cell protein representing a component of DNA polymerase-δ auxiliary protein, a highly conserved protein involved in DNA synthesis, and is produced in all phases of the cell cycle except the resting (G_o) phase (Kurki et al. 1986; Kumaraguruparan et al. 2006; Martin and Johnston 2006). It is a multifunctional protein producing a staining pattern that is generally confined to the nuclei of actively proliferating cells such as those in the stomach, small intestine and colon (Leong et al. 2002). Cell proliferation is a characteristic feature of cellular division (Ortego et al. 1994; Vihtelic et al. 2006). Higher levels of expression of this protein are observed in malignant cells and in inflammatory conditions compared with normal cells (Leong et al. 2002). This cell protein has been detected in mammals, prawns, fish and some higher plants (Suzuka et al. 1989; Ortego et al. 1994; Lyons-Alcantara et al. 1999; Vihtelic et al. 2006). To elucidate whether PCNA is a universal protein necessary for eukaryotic proliferation, a study was conducted to investigate PCNA homologues in higher plants by Suzuka et al. (1994). The localisation of this protein in rice, soybean and tobacco

indicate that the product of this gene plays an essential role in DNA replication in eukaryotes (Suzuka et al. 1994). An immunohistochemical assay for proliferating cell nuclear antigen (PCNA) identified cells in all active phases of the cell cycle of three fish species (Ortego et al. 1994). The techniques used were initially developed for mammalian studies but adapted to study the presence of the PCNA protein in *Oryzias layipes* (medaka), *Poecilia reticulata* (guppy) and *Gambusia affinis* (western mosquitofish). PCNA positive cells were observed along the primary gill filament, the basal cells of the alimentary tract epithelium, the crypt region of the intestine and the testes.

In a study by Lyons-Alcantara et al. (2002), an antibody to PCNA (PC10) was tested on paraffin sections of the crustacean *Pandalus borealis*. PCNA expression was observed in the germinal layer of the oogonia, oocytes and mature ova. Cells of the hepatopancreas and primary gill filament also stained positively for PCNA. The same authors also observed PCNA positive cells in the hepatopancreas of *Nephrops norvegicus* (Lyons-Alcantara et al. 1999). According to Marigomez et al. (1999) who labelled all epithelial cell types of the hepatopancreas of *Mytilus galloprovincialis*, the capacity to proliferate resides in all cell types in the digestive diverticula.

In the study presented here PCNA was detected in the cell nuclei of abalone tissues using the same clone (PC10) as used by Ortego et al. (1994) and Lyons-Alcantara et al. (1999). Antigens in the nuclei of the reproductive organs of both male and female abalone reacted positively with this antibody (Fig. 2.1.2, Fig. 2.4.2, Fig. 2.4.4). Two PCNA antibodies from two different sources were used in this study as they reported a different degree of species cross reactivity. The first PCNA antibody (DAKO) was

reported to react with antigens in mammalian tissues and the second PCNA antibody (Serotec) was a veterinary antibody reported to cross react with antigens in insects and all vertebrates. Western blot analysis also confirmed the presence of PCNA in abalone tissues and additional molecular weight bands, higher than the 36 kDa specific band were observed in the protein extract of *H. discus hannai* (Fig. 2.8.1, Fig. 2.8.2). Cells of the gonad are constantly undergoing cell proliferation giving rise to new cells thus explaining the presence of PCNA in these areas. The digestive gland of molluscs is a multifunctional complex of tubules that are involved in the absorption of digested food, the secretion of digestive enzymes, nutrient storage, algal toxin storage and the release of indigestible food to be eliminated (Voltzow 1994). The hepatopancreas is thus a highly efficient organ with many cells constantly undergoing proliferation in order to carry out its many functions. The results obtained here corroborate previous studies on PCNA expression in invertebrates, signifying that PCNA is a highly conserved protein amongst animals and is a vital element in cell proliferation.

2.4.2 Cytokeratins (MNF and AE1)

This study also demonstrated cytokeratin expression in abalone tissues using broad-spectrum antibodies cytokeratin MNF 116 (5, 6, 8, 17, 19) and cytokeratin AE1 (9, 10, 13, 14, 15, 16, 19). Keratins are intermediate filaments of the cytoskeleton of epithelial cells and they have a structural role in tissues. Intermediate filaments (IFs), which are abundant cytoskeletal structures in most vertebrate cells, play a role in the maintenance of cell type specific architecture and are related to cellular resistance against mechanical stress (Markl et al. 1989; Karabinos et al. 1998; Kumaraguruparan et al. 2006). The IF proteins represent a large multigene family and in human tissues they comprise three nuclear lamins, three neurofilament polypeptides, the glial filament protein (GFP),

desmin, vimentin, peripherin and at least 29 different cytokeratins. The IF proteins play a major role in cell typing and in cell characterisation (Markl et al. 1989; Kumaraguruparan et al. 2006).

A monoclonal antibody that reacts with all classes of intermediate filaments such as glial filaments of astrocytes, neurofilaments in axons, tonofilaments in epithelial PtK2 cells, and intermediate filaments in fibroblasts, was produced by Pruss et al. (1981). Their antibody binds to a protein with a molecular weight of 66 kDa suggesting its presence as a major component of all intermediate filaments and the idea that all intermediate filaments share a common antigenic determinant.

Bartnik and Weber (1989) screened all invertebrate phyla and some early chordates for intermediate filaments using electron microscopy and immunofluorescence with the monoclonal antibody IFA (Intermediate Filament Antigen). According to their results, IF filaments were found in nearly all phyla save the arthropoda and were distributed throughout the epithelia, muscle, glial and neuronal cells. Diogo et al. (1994) was the first study to characterise invertebrate cytokeratins and more recently cytokeratins have been investigated in the prawn and shrimp (Lyons-Alcantara et al. 1999; Lyons-Alcantara et al. 2002). In this study the distribution of cytokeratins in abalone tissues was analysed using two antibodies. Cytokeratin MNF recognises proteins of molecular weight 45-56.5 kDa and displays a broad range of reactivity with human epithelial cells, whether they are simple squamous or stratified (Leong et al. 2002). Cytokeratin AE1 recognises proteins with molecular weights of 56.5, 50, 48 and 40 kDa. The proteins that are thus being targeted by these two antibodies are in the same molecular weight range. There is obviously some degree of overlap in reactivity. Both antibodies react

with normal human epithelia and their neoplasms showing a broad range of reactivity (Leong et al. 2002). Out of four cytokeratin antibodies initially tested in this study, only MNF 116 and cytokeratin AE1 reacted with cytoplasmic proteins in the intestinal epithelia of abalone (Fig. 2.2.2, Fig. 2.5.2). These results were reinforced by western blot analysis and while AE1 produced a specific band of 56.5 kDa in abalone samples, western blotting with cytokeratin MNF revealed many protein bands in abalone tissues indicating numerous cytokeratins, some of which were very distinct in the *H. tuberculata* sample (Fig. 2.8.3, Fig. 2.8.4). With cytokeratin AE1 a band of 56.5 kDa was demonstrated through western blotting corresponding to cytokeratin 10 which is the first report of this cytokeratin in abalone tissues.

The immunohistochemistry of cytokeratins has been researched quite extensively in fish with cytokeratin AE1/AE3 reacting positively with epithelial cells in many fish tissues (Markl and Franke 1988; Markl et al.1989; Bunton 1993). In this study, the combined AE1/AE3 (AE1/3) antibody was also applied to abalone tissues and no staining was detected but staining with AE1 (AE1) was observed. The AE1 antibody recognises type I keratins, cytokeratin 10 which was confirmed in abalone tissues by western blotting is a type I cytokeratin. The AE3 antibody recognises type II keratins which were not detected in abalone tissues.

2.4.3 Vimentin

Vimentin is an intermediate filament component of the cytoskeleton with a molecular weight of 58 kDa. Expressed in virtually all mesenchymal cells, it forms a wavy network of filaments in the cytoplasm of fibroblasts and other connective tissue cells (Leong et al. 2002; Bhosle et al. 2006).

Vimentin has been shown to produce a cytoplasmic pattern of staining in the invertebrate *Drosophila melanogaster* (Walter and Biessman 1984) and has been recognized as a cytoskeletal constituent of many fish tissues (Nelson and Traub 1982; Gyoeva et al. 1987; Dunlap et al. 2006) and invertebrate tissues (Karabinos et al. 1998). Walter and Biessman (1984) used an antibody raised against a protein extracted from *Drosophila* tissue culture cells with a molecular weight of 46 kDa which was also shown to react with vertebrate cells. This antibody was tested on tissues from three invertebrates, *Drosophila melanogaster* (fruit fly), *Neoaplectana carpocapsae* (nematode) and *Paramecium tetraurelia* (unicellular protozoan) and all three organisms bear vimentin-like proteins.

Two different clones of vimentin antibody were tested on abalone tissues in this study. Although the vimentin clone VIM 3B4 has been reported to cross react with *Xenopus laevis* (African clawed frog) vimentin (Herrmann et al. 1989), a positive reaction was not observed in abalone tissues. In this study the V9 clone (57 kDa) detected vimentin in the kidney of one abalone species, *Haliotis tuberculata* and a granular cytoplasmic staining pattern was observed (Fig. 2.6.2) which was confirmed by western blotting with bands in the 57 kDa range but more definite bands were observed at 83 kDa (Fig. 2.8.5) in the *H. tuberculata* protein extract. Markl et al. (1989) used gel electrophoresis, immunostaining and immunoblot assays to identify vimentin in rainbow trout tissues. They concluded that the identified **cyto**skeletal protein is homologous to mammalian vimentin. Some studies have proposed that vimentin may influence protein synthesis but its conservation through species indicates the importance of this protein (Lazarides 1980; Walter and Biessman 1984).

2.4.4 Neuron Specific Enolase (NSE), Neurofilament and S-100

Three different antibodies were employed to detect elements of the nervous system of abalone in this study, NSE, neurofilament and S-100. Neuron specific enolase is a molecular marker for neurons and for central and peripheral neuroendocrine cells (Schmechel et al. 1978; Burleson et al. 2006). It is a highly glycolytic iso-enzyme of the enolase γ-γ dimer found in neurons and neuroendocrine cells (Leong et al. 2002). Antibodies to this enzyme react with neurons and ganglion cells in human tissues (DAKO specification sheet). It is highly sensitive but not very specific (Leong et al. 2002). Neurofilament is an intermediate filament composed of three different subunits of different molecular weights (70, 150, 200 kDa) and is found in neurons of the central and peripheral nervous systems. Neurofilament is a good indicator of neuronal distribution and innervation (Barr et al. 2002; Leong et al. 2002). S-100 is a low molecular weight neural cell protein that is widely expressed in most tissues indicating its role in basic cell function (Ellis 2002; Leong et al. 2002).

The presence of NSE immunoreactivity in neuronal cell bodies in the brain and retrocerebral complex of the insect *Locusta migratoria* was demonstrated by Vullings et al. (1989). Endo and Endo (1988) showed that S-100 is present in the brain neurosecretory cells of invertebrates with particular emphasis on insects and earthworms. This antibody was evaluated in this study but no cross reactivity was observed in abalone tissues. Nerve cells have more recently been characterised in *Haliotis asinina* Linnaeus using antibodies developed that react with serotonin and FMRF-amide neurotransmitters (Panasophonkul et al. 2004).

Neurofilament and S-100 were not detected in abalone tissues in this study. NSE was demonstrated in the connective tissue of the digestive gland which would correspond to the nerve supply for digestion (Fig. 2.3.2). The prosobranch body is entirely innervated and nerves from the buccal ganglia pass to the walls of the oesophagus, stomach and other visceral organs (Voltzow 1994). The staining therefore is considered to be detecting an enolase enzyme present in the intestinal ganglia reflecting innervation. Western blot analysis did not confirm the presence of NSE in abalone tissues owing to non-specific binding of the antibody.

2.4.5 Laminin

Laminin is an embryonically expressed protein that is essential for basement membrane assembly and is one of the most ancient proteins within extracellular matrices, essentially in the basement membranes, and is highly conserved across species (Yurchenco and Wadsworth 2004). It is the first ECM (Extra-Cellular Matrix) molecule to be expressed during embryogenesis in vertebrates and invertebrates (Cooper and McQueen 1983; Martin and Timpl 1987; McCarthy et al. 1987; Montell and Goodman 1989). It is the most abundant glycoprotein in the basement membrane and is both a structural and biologically active component (Martin and Timpl 1987). Developmental and genetic studies have shown that laminins are the only structural components essential for basement membrane assembly (Yurchenco and Wadsworth 2004).

Studies on invertebrate laminins have employed immunofluorescent and immunohistochemical techniques to identify these proteins (McCarthy et al. 1987; Sarras et al. 1994). McCarthy et al. (1987) reported their identification of a sea urchin embryonic extracellular matrix protein from basal lamina preparations. According to

their electron microscope results, the Mab BL1 basal lamina antigen is structurally related to the vertebrate extracellular matrix protein laminin which is useful in understanding the organisation structure and function of the basal lamina. Sarras et al. (1994) investigated the extracellular matrix of the cnidarian, *Hydra vulgaris* revealing the highly conserved nature of laminin and the critical role of ECM components during *Hydra* development. In a study by Montell and Goodman (1988) the similarity of Drosophila laminin with the mouse and human complexes in subunit composition, domain structure and amino acid sequence was demonstrated.

Laminin was detected in abalone using immunohistochemistry underlying the epithelium of the foot in this study. Its presence indicates the highly conserved nature of this structural protein, being present in many species from invertebrates to vertebrates (Sarras et al. 1994; Zhang et al. 1994; Yurchenco and Wadsworth 2004). In abalone this protein serves to maintain the structural conformation of the tissues. Western blot analysis was not performed with laminin as cell lines do not usually produce laminin, and in this study only a small number of basement membranes expressed this protein with immunostaining (Fig. 2.7.2).

The use of mammalian and veterinary antibodies in this study has produced interesting results. They give an indication of the molecules involved in the structural, functional and proliferative framework that makes up the tissues of abalone. All antibodies produced positive staining patterns in both species *H. discus hannai* and *H. tuberculata* with the exception of vimentin which only produced a positive reaction with *H. tuberculata* and stronger bands were also observed with this antibody in western blot trials (Fig. 2.8.5). With the PCNA western blot, additional bands were observed in the

H. discus hannai protein extract. But with cytokeratin MNF and vimentin, the *H. tuberculata* protein extract produced additional stronger bands perhaps indicating a difference in the conformation of proteins between these two species.

Alteration of these proteins may be of interest in studying pathogenic diseases of abalone. Proteins are often upregulated or downregulated in diseased conditions, so the monitoring of protein concentrations or levels could be useful in disease diagnosis or in assessing the stage of disease. This is where antibodies could be developed that are specific for proteins in abalone species as there are few commercial antibodies that react with abalone proteins. In this study twenty four antibodies (Table 2.1, Table 2.2) were tested on abalone tissues and positive results were only obtained with seven antibodies. This indicates that the proteins targeted by the antibodies were not present in abalone tissues or that the antibodies were not species-specific or sensitive enough to detect these proteins. Such negative results indicate the need for the development of antibodies to shellfish antigens if this technique is to be used in shellfish pathology.

Proteins vary from species to species and may have slightly different conformations despite having the same antigenic determinant. The molecular weight of a human protein may be slightly different to the molecular weight of its counterpart in other species, rendering it difficult to absolutely confirm the presence of the same protein in a range of different species. The key findings of this study are that PCNA, cytokeratins, NSE, vimentin and laminin are important biomarkers of proliferation and differentiation in abalone species. The results of this work contribute to an understanding of the biological composition of this shellfish genus and reinforce the results of other studies that suggest some proteins have been conserved throughout evolution.

CHAPTER 3

ANALYSIS OF FUNCTIONAL ENZYMES, CARBOHYDRATES, LIPIDS, PIGMENTS AND MINERALS IN THE ABALONE SHELLFISH (*HALIOTIS DISCUS HANNAI* AND *HALIOTIS TUBERCULATA*) BY HISTOCHEMISTRY.

3.1 INTRODUCTION

Histochemical methods permit the identification of cell type and cellular constituents including carbohydrates, lipids, proteins, pigments and minerals (Drury and Wallington 1980; Bancroft 2002). Using histological techniques it is possible to demonstrate tissue morphology through dye staining, functional characteristics through histochemical and immunohistochemical techniques and tissue ultrastructure through electron microscopy. There are a myriad of histological and histochemical staining techniques available for the demonstration of tissue structures and cellular components (Kiernan 1999). Despite the advent of genetic techniques, histological techniques have maintained their role as the major diagnostic methods for most histopathological investigations.

The use of histology in molluscan studies has long been established, as it yields invaluable information about the biology and the cellular framework of these invertebrates (Hyman 1967; Ruppert and Barnes 1994; Brusca and Brusca 2003). Certain molluscs such as oysters, clams and mussels have been the subject of much investigation due to their global economic value while other molluscs remain relatively under-investigated. Most studies tend to focus purely on histomorphology (Johnson et al. 1996). As molluscan species gain popularity and as aquaculture becomes increasingly important, species-specific information will be essential, supporting more research into marine animals.

The histology of abalone organs and tissues has been investigated to a limited extent, (Stephenson 1924; Crofts 1929; Bevelander 1988; Voltzow 1994; Chitramvong et al. 2002) yet little information is available on the functional phenotype of this shellfish. Studies conducted on this shellfish genus tend to be sporadic and are usually restricted

to specific organs as opposed to a complete analysis of the entire animal (Bolognani-Fantin and Ottaviani 1981; Chitramvong et al. 2002; Wanichanon et al. 2004). Most of the histological surveys of abalone shellfish were conducted in the 1920s, but in 1988 Bevelander thoroughly described the histology of the genus *Haliotis* and this remains the most up to date histological analysis of this genus. The histology, physiology and anatomy of the Prosobranchia were researched and studied by Voltzow (1994). In this study the prosobranchs were described in general, with some species-specific references that cited both *Haliotis tuberculata* and *Haliotis discus hannai*. Bolognani-Fantin and Ottaviani (1981) comparatively assessed the histochemistry of the hypobranchial gland of three different prosobranchs living in different habitats. In 2002 Chitramvong et al. performed a histological analysis of the pallial organs of *Haliotis asinina* Linnaeus and a more recent publication investigated the histology of the hypobranchial gland and gill of *Haliotis asinina* Linnaeus (Wanichanon et al. 2004). While abalone histomorphology has been studied, a more detailed analysis of cellular and functional constituents has not yet been conducted as very few histochemical techniques have been performed on these organisms. In this present study the aim was to systematically analyse the distribution of carbohydrates, lipids, enzymes, pigments and minerals in the organs of *Haliotis tuberculata* and *Haliotis discus hannai*.

3.1.1 Physiological Enzymes in Shellfish

Enzymes are vital components of every biological system and are ubiquitous in all living organisms (Lehninger 2005). The maintenance of life depends on a complex network of chemical reactions that are catalysed efficiently and effectively by specific enzymes. Enzyme histochemistry gives an insight into cell function and the type of metabolic activity that occurs within cells. This contributes significantly to studying the

pathology of organisms, as the absence of enzyme activity is often an indication of cellular dysfunction. There are over one hundred methods for the identification of enzymes described to date (Bancroft 2002). These include methods for the identification of amylases, phosphatases, esterases, lipases, oxidases, peroxidases, proteases and specific enzymes such as ATPase, carbonic anhydrase, cholinesterase, peptidase, catalase, and cellulase to name but a few of the more commonly occurring enzymes within the mollusca (Picos-García et al. 2000; Tengjaroenkul et al. 2002; Bonacci et al. 2004; Luna-González et al. 2004; Ong and Johnston 2006).

Phosphatases, esterases and peptidases are enzymes that perform both specific and general functions in all species. In shellfish they are involved in a range of activities from digestion and sexual differentiation to involvement in the molluscan immune response (Carballal et al. 1997; Mikhailov et al. 1997; García-Carreño et al. 2003). These enzymes are usually present in all tissues while enzymes such as carbonic anhydrase and acetylcholinesterase perform more organ-specific functions.

Hydrolytic enzymes (esterases, phosphatases, proteases, lipases and glycosidases) participate in the destruction of microorganisms inside and outside the hemocytes of shellfish. The function of enzymes in hemocytes and their associated role in the immune response of bivalve molluscs has been investigated in numerous studies (Carballal et al. 1997; Wootton and Pipe 2003; Luna-González et al. 2004). Granulocytes and agranulocytes have been shown to exhibit phagocytic properties resulting in the release of lysosomal enzymes that function in non-self recognition, the encapsulation of foreign bodies and cell death (Carballal et al. 1997; Wootton and Pipe 2003). Both Carballal et al. (1997) and Wootton and Pipe (2003) demonstrated high levels of degradative

enzyme activity in bivalve granulocytes and lower levels of activity in agranulocytes indicating that these hemocyte subtypes serve different functions.

The role of enzymes as biomarkers has become an important area in environmental research (Mora et al. 1999; Galloway et al. 2002; Bonacci et al. 2004). Enzymes are sensitive to pollutants and toxins and the modification of enzymatic activity within an organism can act as an indicator of its surrounding environmental quality.

Research conducted on the characterisation of enzymes in abalone tissues has been very limited but abalone digestive enzymes have been researched (Serviere-Zaragoza et al. 1997; Picos-García et al. 2000; Saitongdee et al. 2004). In abalone culture facilities, artificial diets that are nutritionally suitable and cost-effective are crucial to the success of the industry. Abalone are herbivorous animals and use enzymes to break down the structural polysaccharides of the algae they ingest (Picos-García et al. 2000). To develop specialised diets, knowledge of abalone digestive enzymes is required and the role of digestive enzymes in abalone has been well researched (Serviere-Zaragoza et al. 1997; Hernandez-Santoyo et al. 1998; Picos-García et al. 2000; García-Carreño et al. 2003).

Various enzymatic techniques have been used in the study of shellfish including histochemical staining but the majority of investigations have focused on the extraction and purification of enzymes allowing for the characterisation and quantification of these proteins (Serviere-Zaragoza et al. 1997; Hernandez-Santoyo et al. 1998; Picos-García et al. 2000). Voltzow (1994) performed enzyme histochemical techniques on prosobranch tissues for selected enzymes such as carbonic anhydrase, leucineaminopeptidase,

alkaline phosphatase and acid phosphatase. These enzymes were located in the foot, mantle and digestive system of *Pomatias elegans* (land winkle), *Patella vulgata* (common limpet), *Polinices lewisii* (moon snail) and *Nassarius obsoletus* (eastern mud snail) but were not studied in the Haliotidae. In this study the distribution of the enzymes listed above will be investigated in *Haliotis* spp.

3.1.2 Carbohydrates in Molluscs

Carbohydrates are present in multiple macromolecules in tissues playing a central role in nutrition and as structural components. They can be divided into complex and simple carbohydrates but with tissue carbohydrate demonstration, the two major entities to be considered are mucins and glycogen. Glycogen is a polysaccharide that plays a major role in energy provision as it is the principal storage form of glucose, it is widely distributed in animal tissues and is preserved in paraffin sections. Mucins (glycosaminoglycans/mucosubstances) are the other major group of large carbohydrates and are biochemically categorised into acidic or neutral mucins occurring in mucus, saliva and functioning in secretion and protection (Totty 2002). While there are no subdivisions to the neutral mucins, the acid mucins can be divided into many categories. Firstly the strongly sulphated acid mucins stain at a low pH and are found in epithelial and connective tissues. Connective tissue acid mucins are PAS (Periodic Acid Schiff) negative but acid mucins in the epithelium are PAS positive. Weakly sulphated mucins are epithelial in type staining at a higher pH than strongly sulphated mucins. Carboxylated sialomucins are epithelial in origin and can be enzyme labile or resistant with respect to sialidase. Sulphated sialomucins have a similar reaction to weakly sulphated mucins that can be eliminated with sialidase pre-treatment. Hyaluronic acid is

another sulphated sialomucin that occurs in connective tissue and is formed by fibroblasts. Both hyaluronic and sialic acids stain at similar pH levels (Totty 2002).

To identify carbohydrates, polysaccharides and mucosubstances, histochemical techniques such as the PAS reaction and dye staining methods using alcian blue are the most commonly used (Totty 2002). The PAS technique stains for glycogen, neutral mucins and some epithelial acid mucins. Alcian blue stains all acid mucins. The pH or magnesium concentration of the alcian blue solution is often varied to identify and separate different acid mucins (Totty 2002). Alcian blue and PAS are commonly used in a single stain to simultaneously demonstrate both acid and neutral mucin.

Carbohydrates are frequently studied in molluscs as they form a major component of the natural diet and have an important role in nutrition, particularly in the provision of energy. Abalone are organisms that survive primarily on carbohydrate rich diets, feeding mostly on algae, however both carbohydrates and lipids comprise the principal energy stores in these organisms. Histochemical methods have been used to study carbohydrates such as glycogen, acid mucins and neutral mucins in the epithelial tissues of the various organs of abalone such as the digestive system, the foot, the gill, and the hypobranchial gland, using the PAS and alcian blue dye staining techniques (Bevelander 1988; Voltzow 1994; Wanichanon et al. 2004).

The sole of the foot is extremely glandular and full of mucocytic cells that secrete mucins to ease movement of the foot over the substrata (Voltzow 1994). In addition, the hypobranchial gland is essentially comprised of mucus secreting cells that produce different types of acid mucins, that when released keep the interior of the mantle cavity

clean in turn protecting the visceral organs (Wanichanon et al. 2004). In this study the carbohydrates of all organs of *Haliotis* spp. will be identified and further sub-classified using specialised staining techniques.

3.1.3 Lipids in Shellfish

Lipids are found naturally within an organism usually as stores for energy production and are classified by their insolubility in water (Jones 2002). As aforementioned these fats include true fats, phospholipids, waxes, cholesterols and hydrocarbons. Lipids are classified further based on solubility in solvents (Jones 2002). Simple lipids include fats, oils and waxes which are neutral esters of glycerol with saturated and unsaturated fatty acids. Simple lipids are found as energy stores in adipose tissue and waxes are found in both plants and animals. Compound lipids are found in the central nervous system (CNS) and consist of a fatty acid, alcohol and a nitrogen or phosphorus group. Derived lipids arise as a result of hydrolysis of simple and compound lipids such as cholesterols, bile acids and sex hormones (Jones 2002). Fatty constituents can be identified histochemically in fresh tissue sections using staining techniques that can portray each lipid group. Oil soluble dyes are the most commonly used methods for identification. Oil Red O stains most lipids and is a good method for general screening. It is a popular dye owing to its deep red staining properties and it stains neutral lipids including triglycerides, cholesterol, cerebrosides and phospholipids (Jones 2002). Of the sudan dyes, Sudan Black B is the most sensitive lipid stain. It is a good general lipid indicator staining unsaturated lipids, cholesterol esters, triglycerides and phospholipids.

Papers devoted to lipids and lipid composition in molluscs have been primarily concerned with bivalves and gastropods. Lipids are quite significant in molluscan

nutrition and play an important role in providing concentrated energy (Pillsbury 1985; Delaunay et al. 1991; Mai et al. 1995; Gordon et al. 2006). Most investigations on the archaeogastropods have been concerned with sterols and the sterols of *Haliotis* consist mainly of cholesterol (Voogt 1972; Dunstan et al. 1996).

Lipids in abalone have been researched with respect to dietary intake i.e. using chemical analyses, the carbohydrate to lipid ratio of abalone was established (Thongrod et al. 2003; Viera et al. 2005) and the lipid composition of the hepatopancreas-gonad complex of a gastropod was also studied (Beers et al. 1995). Mai et al. (1995) researched the role of dietary lipid in *Haliotis tuberculata* and *Haliotis discus hannai*. They designed six formulated diets with a series of graded lipid levels and found no significant differences in the survival of abalone fed the artificial diets. However better growth was observed with *H. discus hannai* fed diets with 3.11-7.09% lipid while *H. tuberculata* grew more efficiently with 3.11-5.15% lipid in their diets. Dunstan et al. (1996) analysed the foot of *Haliotis laevigata* and *Haliotis rubra* for fatty acid and sterol composition. They extracted lipid from tissues and discovered that the major sterol in all samples of abalone muscle was cholesterol irrespective of diet but elevated levels of 24-methylcholest-5-enol and 24-ethylcholest-5-enol were due to artificial diets. Most studies on lipids in molluscs use extraction for analysis, so in effect lipid histochemistry is a rarely used technique.

3.1.4 Pigments and Minerals in Molluscs

Naturally pigmented compounds are often encountered in microscopic preparations and may be significant in normal physiological function (e.g. haemocyanin) or in pathological disease (e.g. heavy metal accumulation) (Voltzow 1994; Churukian 2002).

Pigments can be divided into three main categories: endogenous, exogenous and artefact (Churukian 2002). Endogenous pigments are produced within the organism itself. Known molluscan pigments include haemocyanin and myoglobin, which are both respiratory pigments. Haemocyanin is found in blood while myoglobin is found in muscles and in the specialised mouthpart called the odontophore in some molluscs (Voltzow 1994). The haemocyanin composition of three prosobranchs (*Rapana thomasiana* (Thomas' Rapa whelk), *Megathura crenulata* (giant keyhole limpet) and *Haliotis tuberculata* (European abalone) was recently compared using gas-liquid chromatography by Idakieva et al. (2004). Significant differences were observed in the three prosobranchs studied. The two structural subunits of *R. thomasiana* Hc, *Rt*H1 and *Rt*H2 showed 2.6% carbohydrate content while the *M. crenulata* Hc KLH1 and KLH2 showed 3.4% and 3% carbohydrate content respectively and the Hc of *H. tuberculata* showed 4.5% carbohydrate content.

Exogenous minerals are metals or inorganic salts that are acquired externally. Heavy metal pollution has the potential to be a serious aquacultural ecosystem problem but it can be controlled, in comparison to heavy metal accumulation in the wild which cannot be regulated as easily (Tsai et al. 2006). Shellfish have been used as environmental indicators because of their tendency to accumulate metals and toxins from their surrounding environment. According to a study in Australia, the concentrations of cadmium, copper, iron and zinc were significantly higher in wild abalone populations in comparison to cultured shellfish. This was related to the lower concentrations of metals in the substrates and food of cultured molluscs (Skinner et al. 2004). These metals and other trace metals can be demonstrated by histochemistry. Chemical analyses have been employed to assess the bioaccumulation of heavy metals in abalone (Liao and Ling

1999; Liao et al. 2004; Skinner et al. 2004; Hernández et al. 2006). Few studies have assessed the presence and cellular distribution of pigments in abalone using histochemistry.

In this study the distribution of functional and structural elements including enzymes, carbohydrates, lipids, pigments and minerals will be investigated in *Haliotis tuberculata* and *Haliotis discus hannai*, using a panel of histochemical methods. The distribution of esterases, phosphatases, carbonic anhydrase, peroxidase, leucineaminopeptidase and acetylcholinesterase will be investigated in both species. Distribution and type of carbohydrates including glycogen, neutral and acid mucins, carboxylated and strongly sulphated acid mucins will also be investigated. These results will be related to lipid distribution and pigment and mineral content of the cells and organs of this shellfish genus.

3.2 MATERIALS AND METHODS

3.2.1 Specimen Collection

- On December 8th 2002, 10 abalone shellfish (3 *Haliotis discus hannai* of 5 cm shell length, 4 *H. discus hannai* of 2 cm shell length and 3 *Haliotis tuberculata* of 5 cm shell length) were collected from the Boet Mór shellfish farm, Clifden, Co. Galway, Ireland.
- On January 21st 2004, 14 abalone shellfish (7 *H. discus hannai* of 5 cm shell length and 7 *H. tuberculata* of 5 cm shell length) were collected from the Boet Mór shellfish farm, Clifden, Co. Galway, Ireland.
- On November 9th 2004, 33 abalone shellfish (5 *H. discus hannai* of 5 cm shell length, 8 *H. discus hannai* of 2 cm shell length, 10 *H. tuberculata* of 5 cm shell length and 10 *H. tuberculata* of 2 cm shell length) were collected from the Boet Mór shellfish farm, Clifden, Co. Galway, Ireland

Abalone of 5 cm shell length were approximately 3 years old (adult) and abalone of 2 cm shell length were approximately 1 year old (young). Male and female abalone were collected. Specimens were transported to Dublin in a container of seawater through which air was bubbled. All shellfish appeared healthy upon arrival in Dublin, were immediately placed in 50% alcohol: 50% seawater and prepared for experimental work (paraffin processing or frozen processing).

3.2.2 Abalone Samples – Frozen Tissues

Frozen sections were prepared for all enzyme and lipid staining methods. All specimens were placed in 50% alcohol and 50% seawater for 30 minutes. Samples were immediately dissected and a small piece of each organ was embedded in OCT

compound and snap frozen in liquid nitrogen (-196°C). The frozen blocks were wrapped in aluminium foil and stored at -70°C.

Frozen sections (7 µm in thickness as opposed to previous 5 µm paraffin sections because frozen sections are easier to cut at 7 µm) were cut using a cryostat at -20°C and melted onto APES-coated slides. All frozen sections were air dried at RT prior to fixation for 1 minute in acetone or 10% formalin and subsequent staining.

3.2.3 Sample Preparation for Histochemistry

For carbohydrate, pigment and mineral staining methods, paraffin embedded sections were prepared. After 30 min in absolute alcohol, the molluscs were removed from their shells and placed in Davidson's fixative (Appendix A) for 24 h. An incision was made lengthwise down the foot muscle of each mollusc to allow penetration of the fixative. Individual organs (digestive gland, foot, gill, gonad, hypobranchial gland, mantle and renal organs) were dissected from shellfish of 5 cm in length (adult) and animals less than 2 cm in length (young) were cross-sectioned longitudinally.

3.2.4 Paraffin Processing of Fixed Tissue to Paraffin Wax

All samples preserved in Davidson's fixative were placed on an automated processor (Histokinette) for 18 h and passed through the following solutions: 10% formalin, spirit (95% ETOH), absolute alcohol (x5), xylene (x3) and paraffin wax (x2). Tissues were embedded in paraffin wax and 5 µm sections were cut using a microtome. All sections were set onto adhesive APES-coated slides and incubated in an oven at 56 °C for 2-3 h. Slides were then stained using the histochemical staining methods as outlined below.

3.2.5 Histochemical Methods

Harris' Haematoxylin and Eosin (1900) was the stain of choice for the demonstration of general tissue morphology.

Enzyme detection kits (Sigma-Aldrich, Dublin) were used for the demonstration of acid phosphatase (Catalogue No. 387-A), alkaline phosphatase (Catalogue No. 86-R), α-naphthyl butyrate esterase (Catalogue No. 181-B) and naphthol AS-D chloroacetate esterase (Catalogue No. 91-C). Carbonic anhydrase (Hansson 1967), leucineaminopeptidase (Nachlas et al. 1957), acetylcholinesterase (Filipe and Lake 1983) and peroxidase (Graham and Karnovsky 1966) enzymes were detected using established histochemical methods.

Carbohydrates were identified using the following protocols. Alcian Blue/Periodic Acid Schiff (ABPAS) (Mowry 1956) was used to identify general carbohydrate distribution. Periodic Acid Schiff (PAS) (Schiff 1866; McManus 1946) was used to demonstrate neutral mucins, sialomucins and glycogen. Diastase Periodic Acid Schiff (DPAS) was used to separate glycogen and neutral mucin by treatment with the glycogen digestion enzyme diastase (Totty 2002). The mild PAS technique (Roberts 1977) was used to detect N-acetyl sialomucins. Alcian blue (AB) @ pH 2.5 was used to identify all acid mucins while alcian blue @ pH 0.2 was used to stain strongly sulphated acid mucins (Totty 2002). The methylation/saponification technique (Spicer and Lillie 1959) identified carboxylated mucin, sialomucin and hyaluronic acid.

Neutral lipids were identified using Oil Red O (French 1926). For the identification of pigments the following stains were used: Rhodanine technique for copper (Lindquist 1969; Jain et al. 1978), Perl's Prussian blue (Perls 1867) for iron, Masson Fontana

(Fontana 1912; Masson 1914) for melanin, Alizarin Red S (McGee-Russell 1958) and Von Kossa (Von Kossa 1901) for calcium.

All staining protocols were taken from and Bancroft and Stevens (1990), Bancroft and Cook (1994) and Bancroft and Gamble (2002) (Appendix C – Histochemical Staining protocols). A list of all histochemical stains and what they identify can be seen in Table 3.1.

Positive controls used were: blood smears for esterases, phosphatases and peroxidase enzymes. Hirschprung's intestine (Our Lady's Hospital for Sick Children Crumlin, Dublin) was used for acetylcholinesterase, rat pancreas for carbonic anhydrase and rat kidney for leucineaminopeptidase, (Laboratory rats were obtained from the School of Biochemistry and Immunology, Trinity College Dublin). Rat intestine was used as a positive control for all carbohydrates and mucins. Fresh frozen lard/fatty liver was used as a control for lipids. Rat liver was a positive control for copper and iron, intestine for melanin and kidney for calcium. (Appendix D – positive control images). For verification of positive results, all staining methods were performed on all samples in triplicate.

All staining protocols were tested on both species of abalone, *Haliotis discus hannai* and *Haliotis tuberculata*. However, the best pattern of staining for each method was only chosen for photography, whether it was observed in *H. discus hannai* or *H. tuberculata*. The same results were obtained for each species unless otherwise stated in the results section. For the study on the reproductive system, male and female abalone were compared.

Table 3.1

List of Histochemical Staining Techniques and Components Stained

Category	Staining Technique	Component Stained
Enzymes	Leukocyte acid phosphatase azo-dye based kit (Sigma-Aldrich Inc.)	Acid phosphatase
	Acetylcholinesterase (Filipe and Lake 1983)	Acetylcholinesterase
	Leukocyte alkaline phosphatase azo-dye based kit (Sigma-Aldrich Inc.)	Alkaline phosphatase
	α-naphthyl butyrate esterase (Sigma-Aldrich Inc.)	α-naphthyl butyrate esterase
	Hansson's carbonic anhydrase (metal precipitation technique)	Carbonic anhydrase
	Naphthol AS-D chloroacetate esterase (Sigma-Aldrich Inc.)	Chloroacetate esterase
	Metal chelation technique (Nachlas et al. 1957)	Leucineaminopeptidase
	DAB (diaminobenzidine reaction)	Peroxidase
Carbohydrates	Periodic Acid Schiff	Polysaccharides and carbohydrate-protein complexes
	Alcian Blue/Periodic Acid Schiff	Acid and neutral mucins
	Alcian Blue @ pH 2.5	Acid mucins
	Alcian Blue @ pH 0.2	Sulphated acid mucins
	Methylation-Saponification technique	Carboxylated mucins
Lipids	Oil Red O	Neutral lipids
Pigments & Minerals	Rhodanine	Copper
	Perl's Prussian blue	Iron
	Masson Fontana	Melanin
	Alizarin Red S	Calcium

3.2.6 Microscopic Photography

Histological images were captured using two different types of photography. Digital images were captured using a Canon Power Shot S50 digital camera (Canon Inc., Japan), attached to a Leica DM LS2 microscope (Leica, Germany). Images were captured using the Canon Remote Capture computer programme and saved as jpegs. Photographs were also taken using a manual Wild Leitz MP S52 camera and Wild MPS 46 Photoautomat panel, attached to a Leitz Labor Lux S microscope (Leitz, Heerburg, Switzerland). Photographic films were developed, scanned into a computer and images were saved as jpegs.

3.3 RESULTS

3.3.1 FOOT

The foot tissue of abalone is composed of epithelia, muscle and connective tissue (Voltzow 1994) (Fig. 3.1.1). There are two types of epithelia present in the foot. The sole of the foot has a stratified columnar epithelium (pedal epithelium) underlain by a basement membrane. A single layer of cuboidal epithelium lines the peripheral edge of the foot muscle (peripheral epithelium). The epithelial cells are both ciliated and non-ciliated (Crofts 1929). Associated with the epithelia are goblet cells, sub-epithelial gland cells and sub-epithelial ganglion cells (Crofts 1929). All prosobranch muscle fibres are spindle shaped, unicellular and disorganised with respect to thick and thin filaments (Voltzow 1994). The predominant muscle type is smooth with bundles of muscle fibres wrapped within sheaths of connective tissue. The extracellular matrix of connective tissue surrounding the foot muscle is primarily composed of collagen (Voltzow 1994).

3.3.1 (a) Peripheral Epithelium

Enzymes

Acid and alkaline phosphatases were observed in the simple cuboidal epithelial cells (Fig. 3.1.2, Fig. 3.1.3). The peripheral epithelial cells were positive for the presence of non-specific esterases (Fig. 3.1.4). Peroxidase was identified in epithelial cells of the peripheral epithelium (Fig. 3.1.5). Carbonic anhydrase was detected in the peripheral epithelium (Fig. 3.1.6).

Carbohydrates

Acid mucins were demonstrated in mucous cells in the peripheral epithelia by ABPAS (Fig. 3.1.7). Mucous cells within the epithelial layer stained positively with alcian blue demonstrating acidic mucopolysaccharides and strongly sulphated acid mucins (Fig. 3.1.8, 3.1.9).

Pigments and Minerals

Pigmented granules in the peripheral epithelia were positive for melanin (Fig. 3.1.10) but were negative for iron and copper.

3.3.1 (b) Pedal Epithelium

Enzymes

A similar pattern of staining was observed in this epithelium as the peripheral epithelium. Alkaline phosphatase and acid phosphatase were very abundant enzymes in the foot. Intense expression of both enzymes was detected in the pedal epithelia. Esterase was observed in the pedal epithelial cells of the foot (Fig. 3.1.11). Peroxidase was identified in epithelial cells of the pedal epithelium. Carbonic anhydrase was detected in the epithelial cells of the foot with activity located in the apical regions of the cells.

Carbohydrates

The combined ABPAS technique detected the presence of abundant acid mucins in the epithelium. The surface of the columnar epithelium was rich in neutral mucin (Fig. 3.1.12).

The pedal epithelium of the foot was scattered with both acid and neutral mucins. Mucous cells within the epithelium stained positively with alcian blue indicating the presence of acidic mucopolysaccharides.

Pigments and Minerals

The basement membrane of the epithelium was positive for calcium (Fig. 3.1.13). Iron and copper were not identified in this epithelium.

3.3.1 (c) Sub-epithelial Gland Cells

Carbohydrates

The sub-epithelial gland cells stained intensely blue with ABPAS indicating that they were comprised mainly of acid mucin (Fig. 3.1.14). Occasional cells were PAS positive and are considered to be macrophages. Acid mucins and carboxylated acid mucins were detected in the sub-epithelial gland cells of the foot (Fig. 3.1.15, Fig. 3.1.16).

3.3.1 (d) Sub-epithelial Ganglion Cells

Enzymes

Clumps of sub-epithelial ganglion cells in the muscle expressed esterase activity (Fig. 3.1.11, 3.1.17, 3.1.18).

The surrounding edges of the sub-epithelial ganglia were weakly PAS positive for neutral mucin (Fig. 3.1.12). No staining for acid mucin was observed.

3.3.1 (e) Connective Tissue and Muscle

Carbohydrates

The muscle cells of the foot were PAS positive which is representative of mucin as confirmed by DPAS staining (Fig. 3.1.12). Within the muscle layer are blood vessels and the endothelial layer of these vessels was rich in neutral mucin.

The connective tissue exhibited weak alcian blue staining, indicating the presence of acid mucin in the ground substance (Fig. 3.1.8).

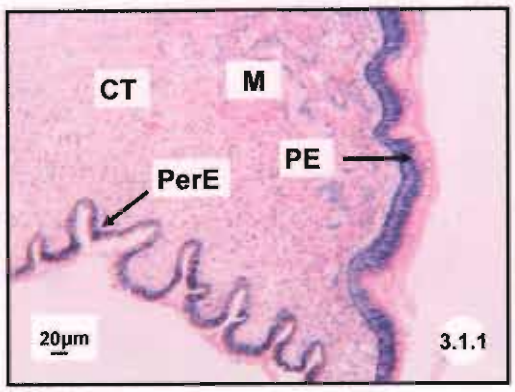

Figure 3.1.1: H&E of foot epithelium, and connective tissue of *Haliotis discus hannai.* (Pedal epithelium PE; peripheral epithelium PerE; muscle M; connective tissue CT) (x200 digital camera).

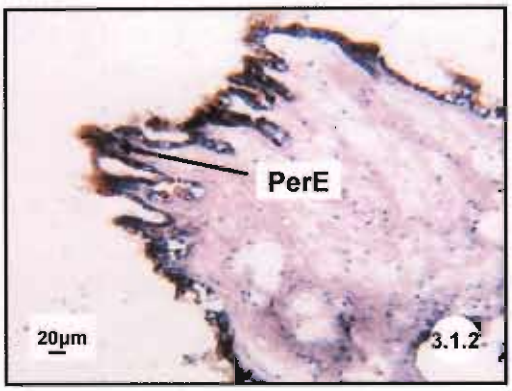

Figure 3.1.2: Acid phosphatase (red/brown) in the peripheral epithelial cells (PerE) of the foot of *H. discus hannai* (x200 digital camera).

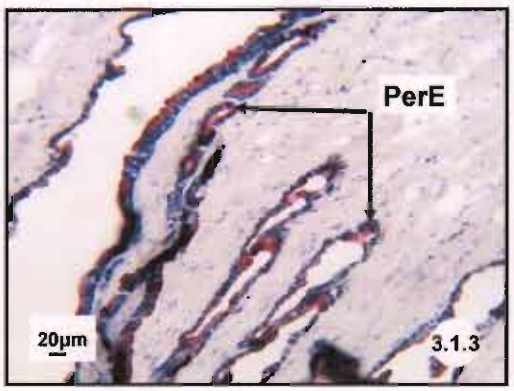

Figure 3.1.3: Alkaline phosphatase (pink) in the peripheral epithelial cells (PerE) of the foot of *H. tuberculata* (x200 digital camera).

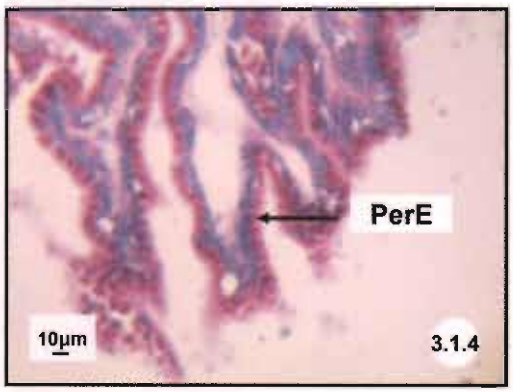

Figure 3.1.4: Chloroacetate esterase (pink) in the peripheral epithelia of the foot of *H. discus hannai* (x400 digital camera).

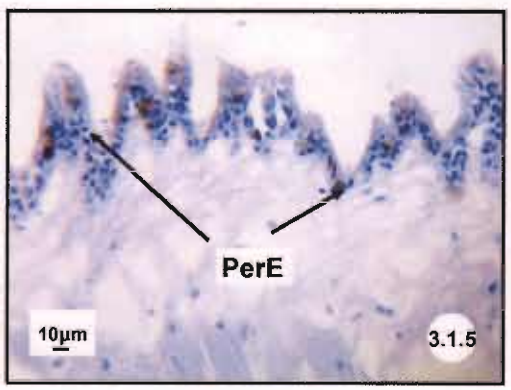

Figure 3.1.5: Peroxidase (brown) in the peripheral epithelia (PerE) of the foot of *H. tuberculata* (x400 digital camera).

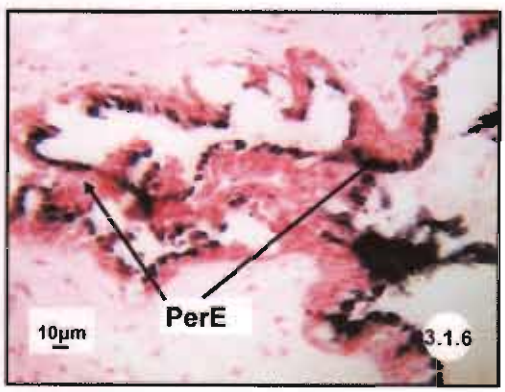

Figure 3.1.6: Carbonic anhydrase (black) in the peripheral epithelia (PerE) of the foot of *H. tuberculata* (x400 digital camera).

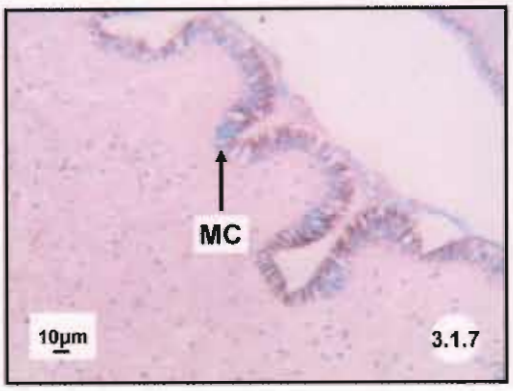

Figure. 3.1.7: ABPAS: Acid mucins (blue) in the mucous cells (MC) of the foot epithelia of *H. discus hannai* (x400 digital camera).

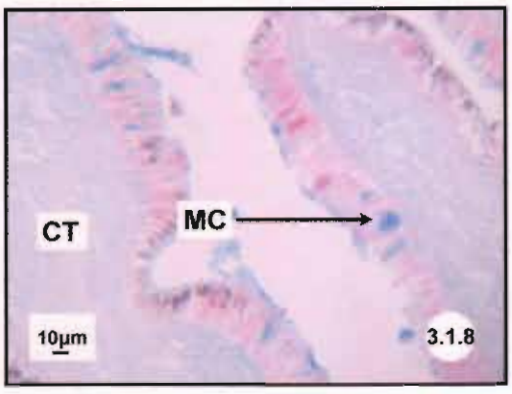

Figure 3.1.8: AB pH 2.5: acid mucins (blue) in the epithelial mucous cells (MC) and connective tissue (CT) layer of the foot of *H. discus hannai* (x400 digital camera).

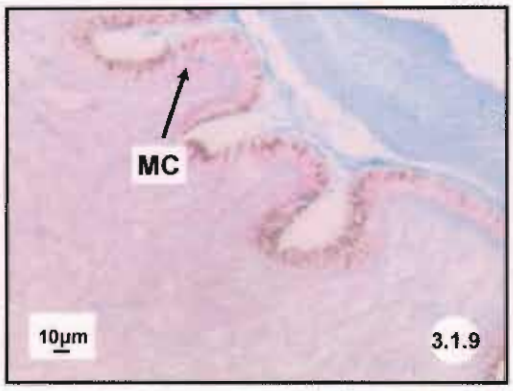

Figure 3.1.9: AB pH 0.2: sulphated acid mucins (blue) in the epithelial mucous cells (MC) of the foot of *H. discus hannai* (x400 digital camera).

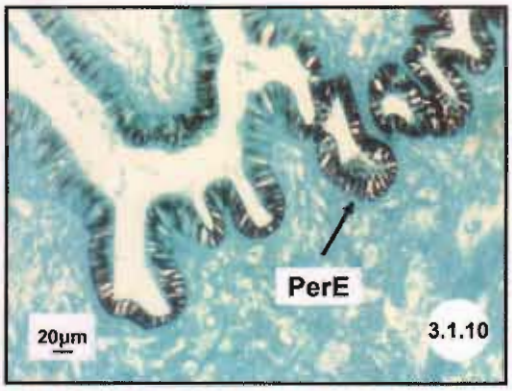

Figure 3.1.10: Masson Fontana: melanin (black) pigments in the peripheral epithelia (PerE) of the foot of *H. discus hannai* (x400 camera).

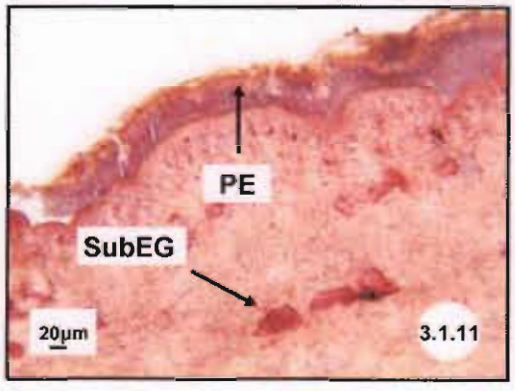

Figure 3.1.11: Alpha naphthyl butyrate esterase (red/brown) in the pedal epithelia (PE) and sub-epithelial ganglion cells (SubEG) of the foot of *H. discus hannai* (x200 manual camera).

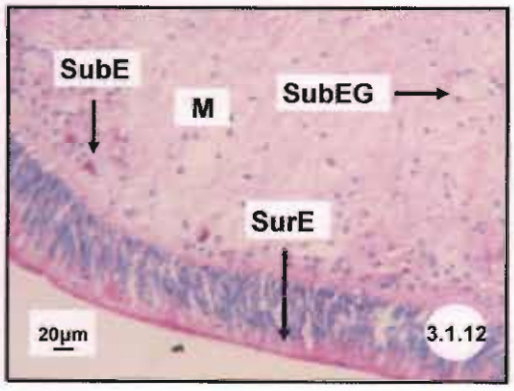

Figure 3.1.12: PAS (magenta) positive surface epithelia (SurE); muscle (M); sub-epithelial cells (SubE) and sub-epithelial ganglia (SubEG) of *H. discus hannai* (x400 manual camera).

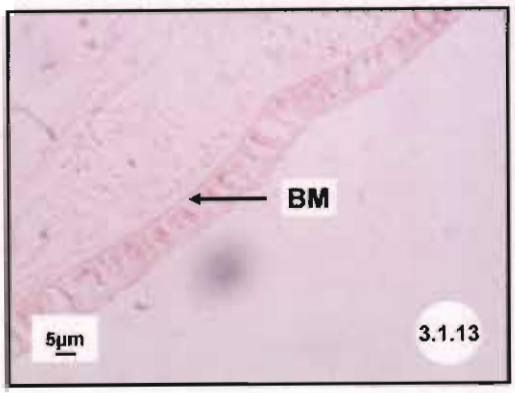

Figure 3.1.13: Alizarin Red S: Calcium (red) positive basement membrane (BM) of the foot of *H. discus hannai* (x1000 oil digital camera).

Figure 3.1.14: ABPAS: Acid mucins (blue) in the sub-epithelial gland cells (SubE) of the foot of *H. discus hannai* (x400 digital camera).

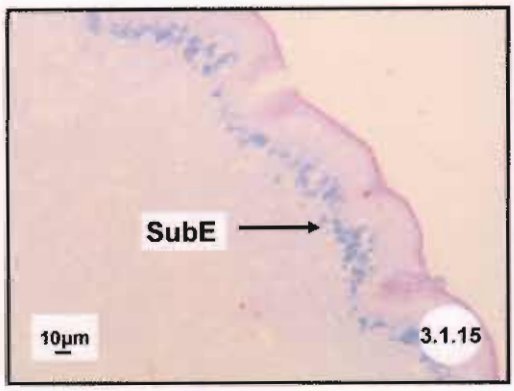

Figure 3.1.15: AB pH 2.5: Acid mucins (blue) in the sub-epithelial gland cells (SubE) in the foot of *H. discus hannai* (x400 digital camera).

Figure 3.1.16: Methylation - Saponification technique: carboxylated mucins (blue) in the sub-epithelial gland cells (SubE) in the foot of *H. discus hannai* (x400 digital camera).

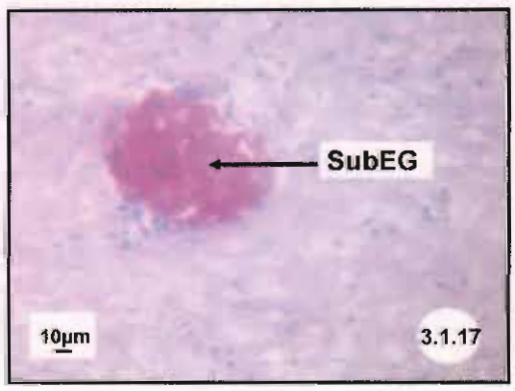

Figure 3.1.17: Chloroacetate esterase (pink) in sub-epithelial ganglion cells (SubEG) of the foot of *H. discus hannai* (x400 digital camera).

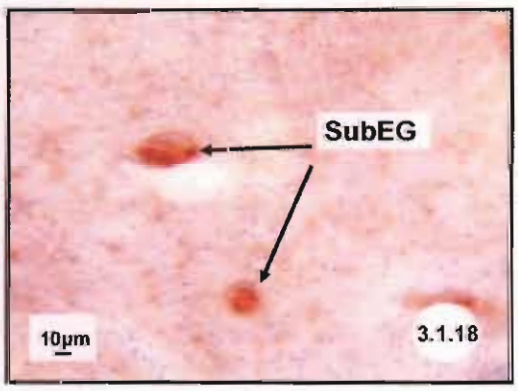

Figure 3.1.18: Acetylcholinesterase (brown) in sub-epithelial ganglion cells (SubEG) in the foot of *H. discus hannai* (x400 digital camera).

3.3.1 (f) Summary of Histochemistry

The pedal and peripheral epithelia expressed high levels of the following enzymes: esterases, alkaline and acid phosphatases, peroxidase and carbonic anhydrase. Both epithelia were negative for the enzymes leucineaminopeptidase and acetylcholinesterase. These two enzymes were not detected in the sub-epithelial gland cells, connective tissue and muscle. The sub-epithelial ganglion cells were positive for non-specific esterases and acetylcholinesterase. Of the carbohydrates, neutral mucins, acid mucins and strongly sulphated mucins were found within the columnar epithelial cells, in sub-epithelial gland cells and in the ground substance of the connective tissue and muscle. No lipid stores were present in the foot. Melanin was identified in the sub-epithelial gland cells, in the pedal and peripheral epithelia. The epithelial basement membrane of the foot was rich in calcium in one *H. discus hannai* sample detected by Alizarin Red S.

3.3.2 MANTLE

The mantle is a fold of tissue lining the shell and covering the viscera (Voltzow 1994). It consists of a layer of loose connective tissue, muscle fibres, blood vessels, nerves and is covered by epithelium. The epithelium of the mantle is usually squamous or columnar but the cells increase in depth towards the periphery where the mantle folds and becomes pleated (Fig. 3.2.1). The mantle bears both ciliated and non-ciliated epithelial cells and mucous cells.

3.3.2 (a) Mantle Epithelium

Enzymes

Acid phosphatase was detected in the apical zones of the columnar epithelium and along the upper surface of the epithelium (Fig. 3.2.2). Alkaline phosphatase was detected in the mantle epithelium (Fig. 3.2.3).

The columnar epithelium of the mantle was positive for alpha-naphthyl butyrate esterase (Fig. 3.2.4). The simple columnar epithelium contained cells positive for chloroacetate esterase with expression in the upper region of the epithelial cells (Fig. 3.2.5). Peroxidase was also detected in the mantle epithelial cells (Fig. 3.2.6).

Carbohydrates

The majority of cells in the mantle epithelium were alcian blue positive for acid mucin with a few PAS positive mucous cells within the columnar epithelium, as confirmed by ABPAS staining (Fig. 3.2.7). Mucous cells within the epithelium stained positively with PAS for neutral mucin (Fig. 3.2.8). The basement membrane of the epithelium was PAS positive for neutral mucin (Fig. 3.2.9). The epithelia were negative for N-acetyl sialomucins but positive for sulphated acid mucins. Acid mucins were abundant in the

epithelia (Figure 3.2.10) and strongly sulphated acid mucins were present in the mantle epithelia (Fig. 3.2.11).

3.3.2 (b) Connective Tissue and Muscle

Enzymes

Alpha naphthyl butyrate esterase was detected in clumps of cells within the mantle connective tissue (Fig. 3.2.12). Chloroacetate esterase was also present in scattered clumps of ganglion-type cells in the connective tissue and muscle. Acetylcholinesterase was detected in the connective tissue just below the epithelial layer (Fig. 3.2.13).

Carbohydrates

The connective tissue of the mantle was weakly PAS positive for mucin reflecting the carbohydrates of the ground substance (Fig. 3.2.8). Alcian blue at both pH 0.2 and pH 2.5 did not detect any acid mucins or sulphated mucins in the connective tissue. N-acetyl sialomucins were not present in the connective tissue of the mantle.

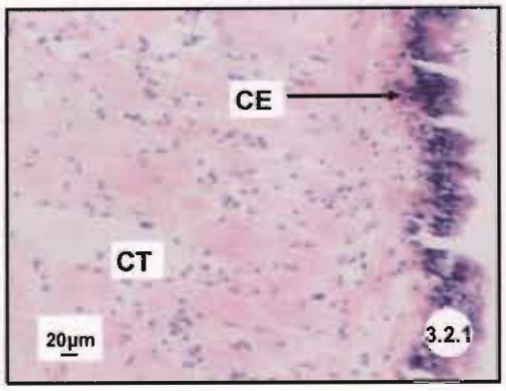

Figure 3.2.1: H&E of mantle columnar epithelia (CE) and connective tissue (CT) in the mantle of *H. tuberculata* (x200 digital camera).

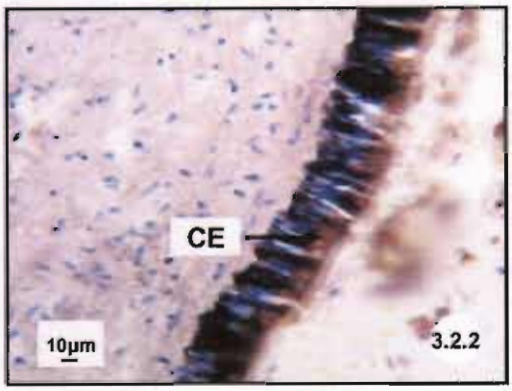

Figure 3.2.2: Acid phosphatase (red/brown) in the columnar epithelia (CE) in the mantle of *H. discus hannai* (x400 digital camera).

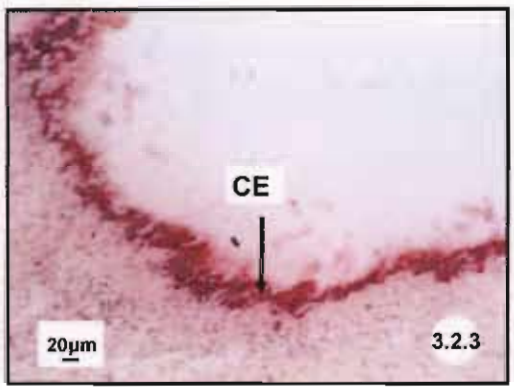

Figure 3.2.3: Alkaline phosphatase (pink) in the columnar epithelia (CE) of the mantle of *H. discus hannai* (x200 digital camera).

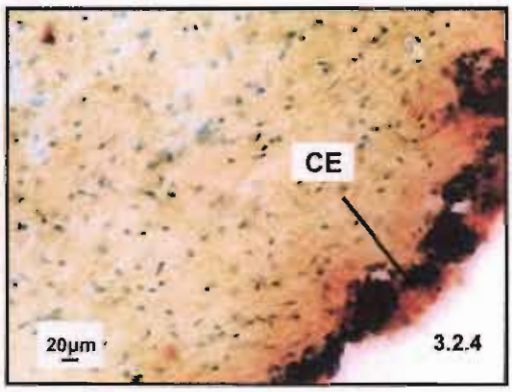

Figure 3.2.4: Alpha naphthyl butyrate esterase (red/brown) in the columnar epithelia (CE) in the mantle of *H. discus hannai* (x200 digital camera).

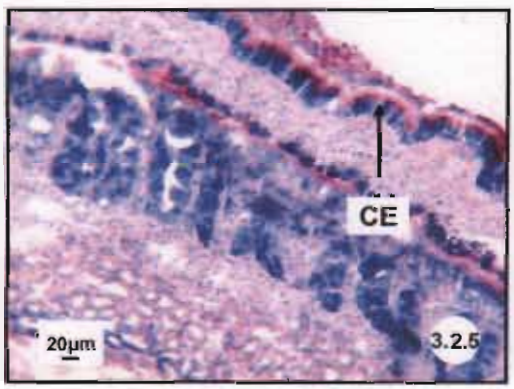

Figure 3.2.5: Chloroacetate esterase (pink) positive columnar epithelia (CE) in the mantle of *H. discus hannai* (x200 digital camera).

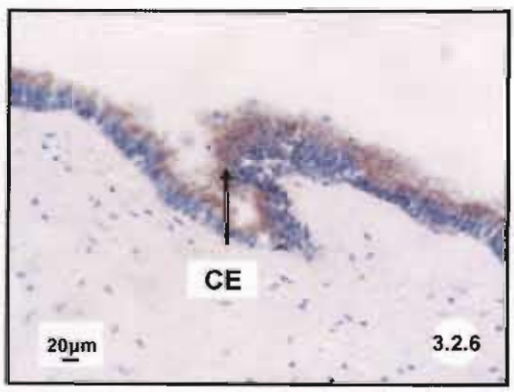

Figure 3.2.6: Peroxidase (brown) in the columnar epithelia (CE) in the mantle of *H. discus hannai* (x200 digital camera).

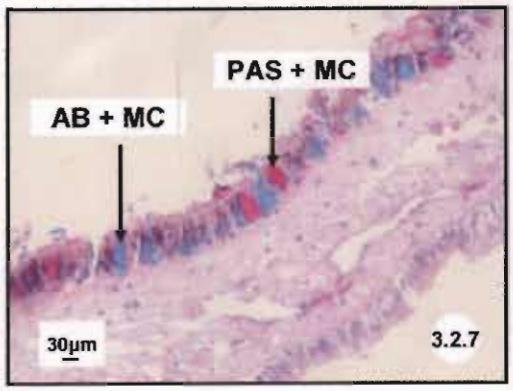

Figure 3.2.7: ABPAS of mantle: Neutral mucins (magenta) and acid mucins (blue) in the mucous cells (MC) of the columnar epithelia in the mantle of *H. discus hannai* (x200 manual camera).

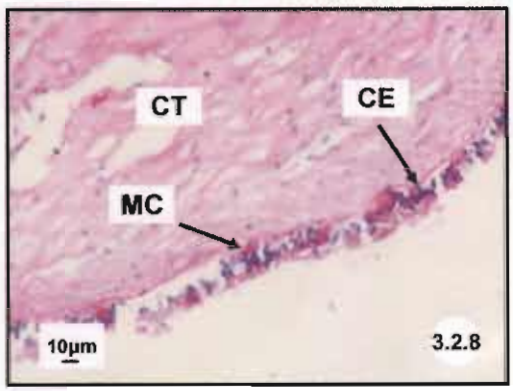

Figure 3.2.8: Periodic Acid Schiff: PAS (magenta) positive connective tissue (CT) and mucous cells (MC) in the columnar epithelia (CE) in the mantle of *H. tuberculata* (x400 digital camera).

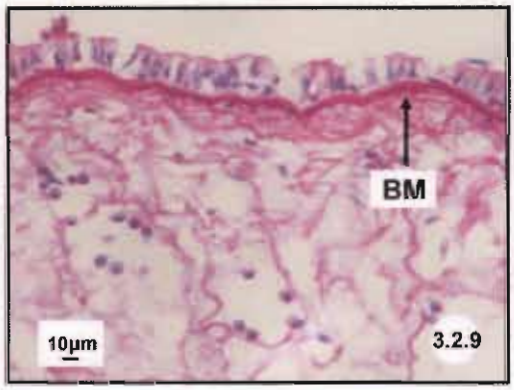

Figure 3.2.9: PAS: PAS positive (magenta) basement membrane (BM) in the mantle of *H. discus hannai* (x400 digital camera).

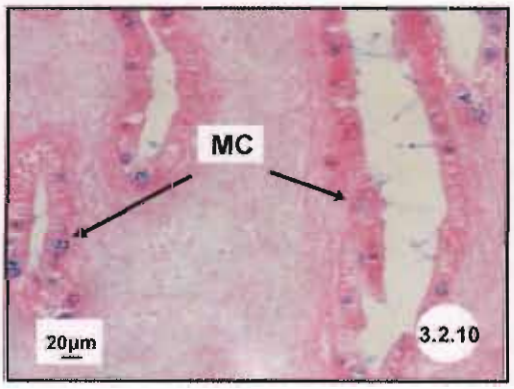

Figure 3.2.10: Alcian blue (pH 2.5): acid mucins (blue) in the mucous cells (MC) of the mantle epithelia of *H. tuberculata* (x400 manual camera).

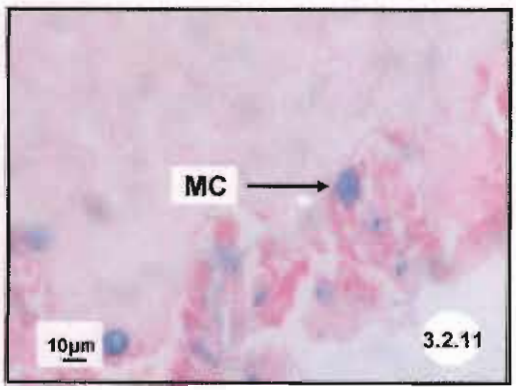

Figure 3.2.11: Alcian blue (pH 0.2): strongly sulphated mucins (blue) in the mucous cells (MC) of the mantle epithelia of *H. discus hannai* (x1000 oil manual camera).

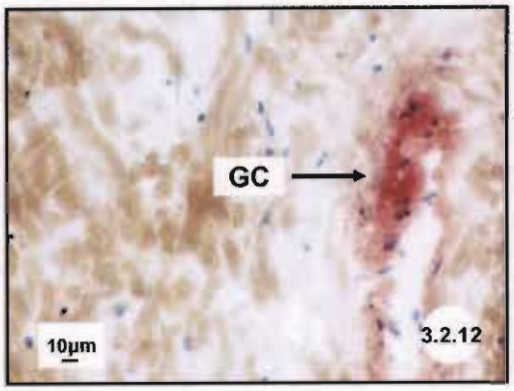

Figure 3.2.12: Alpha naphthyl butyrate esterase (red/brown) in the ganglion type cells (GC) of the mantle connective tissue of *H. tuberculata* (x400 digital camera).

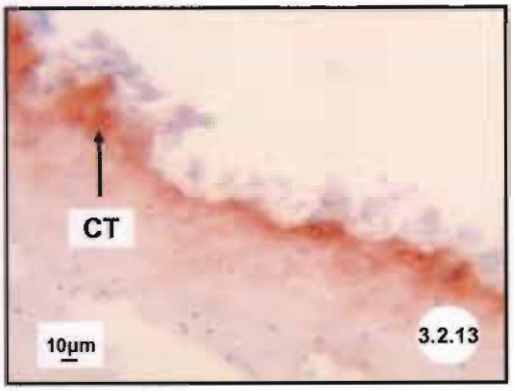

Figure 3.2.13: Acetylcholinesterase (brown) in the connective tissue (CT) of the mantle of *H. tuberculata* (x400 digital camera).

3.3.2 (c) Summary of Histochemistry

Epithelial cells of the mantle were positive for acid phosphatase, alkaline phosphatase, non-specific esterases and peroxidase. The enzymes carbonic anhydrase and leucineaminopeptidase were not expressed in the mantle. Carbohydrates proved to be in abundance in the mantle epithelia and connective tissues, but lipids and pigments were not detected in the mantle tissue and cells.

3.3.3 DIGESTIVE SYSTEM

The digestive tract consists of the oesophagus, crop, stomach, caecum, intestine and digestive diverticulum (digestive gland/hepatopancreas). The digestive tract has been described in detail in section 1.3.2 in Chapter 1. The digestive gland or hepatopancreas of abalone is a large asymmetrical organ that is comprised of a series of tubules/ducts. There are two types of secretory cells in the digestive gland: duct cells and crypt cells (Fig. 3.3.1). The duct cells make up most of the digestive diverticula and are tall and cylindrical in shape resting upon a connective tissue lamina. These cells have numerous clear vacuoles and granules, as well as nuclei located in the apical regions of the cells. The crypt cells are smaller flask shaped cells with basal nuclei and are interspersed between the duct cells (Bevelander 1988). They are dark staining cells that tend to be ciliated and contain large iron granules.

3.3.3 (a) Duct Cells

Enzymes

Focal expression (dot staining) of acid phosphatase was detected in the duct cells of the digestive gland (Fig. 3.3.2). Alkaline phosphatase was also expressed surrounding the digestive gland cells (Fig. 3.3.3). Alpha naphthyl butyrate esterase and chloroacetate esterase were weakly expressed in the duct cells of the digestive gland (Fig. 3.3.4, Fig. 3.3.5). Leucineaminopeptidase was not detected in the duct cells.

Carbohydrates

Acid mucins were expressed in the duct cells of the digestive gland (Fig 3.3.6). The digestive cells towards the lumen were strongly PAS positive indicating neutral mucin (Fig. 3.3.7). The duct cells were alcian blue positive for both acid mucins (Fig. 3.3.8) and strongly sulphated mucins (Fig. 3.3.9).

3.3.3 (b) Crypt Cells

Enzymes

The crypt cells displayed activity for acid phosphatase. This enzyme produced a granular pattern of staining with intense enzyme activity around the pigmented areas of the cells (Fig. 3.3.2). As with the duct cells alkaline phosphatase was localised towards the periphery of the cells (Fig. 3.3.3).

The presence of alpha naphthyl butyrate esterase was assessed and was observed towards the base of the crypt cells located near the pigments (Fig. 3.3.4). Intense chloroacetate esterase staining was also found to be present in the cells around the pigments (Fig. 3.3.5).

Carbohydrates

With ABPAS staining the crypt cells were both AB and PAS negative (Fig. 3.3.6). The crypt cells were weakly PAS positive for neutral mucin around the base of the cells (Fig. 3.3.7).

Lipids and Pigments

Neutral lipids and phospholipids were particularly abundant around the basal regions of the epithelial cells. The pattern of staining was generally large fat droplets around the pigmented areas of the crypt cells with smaller fat droplets distributed above and below the pigments (Fig. 3.3.10).

The pigments in the crypt cells were intensely positive for iron when stained with Perl's Prussian blue (Fig. 3.3.11).

3.3.3 (c) Digestive Tract

Enzymes

Leucineaminopeptidase was confined to the digestive tract of the digestive system. This enzyme was positive around the edge of the epithelium joining the connective tissue

(Fig. 3.3.12). Alkaline phosphatase was observed on the upper surface of the epithelia of the digestive tract (Fig. 3.3.13). Alpha naphthyl butyrate esterase positivity was observed in the digestive tract along the outer edge of the epithelial layer (Fig. 3.3.14). Chloroacetate esterase was located lining the upper surface of the digestive tract epithelia (Fig. 3.3.15).

Lipids

Lipid droplets were observed lining the apical sections of the digestive tract (Fig. 3.3.16).

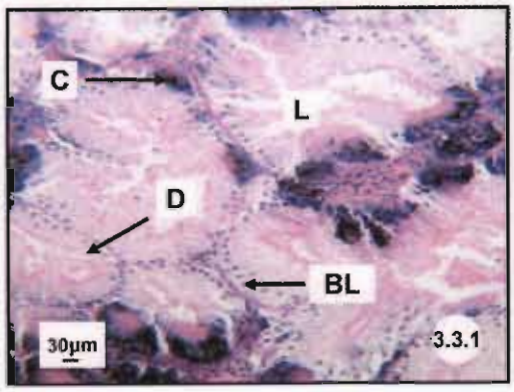

Figure 3.3.1: H&E of the digestive gland of abalone showing duct cells (D), crypt cells (C), basement lamina (BL) and the lumen (L) of *Haliotis discus hannai* (x200 manual camera).

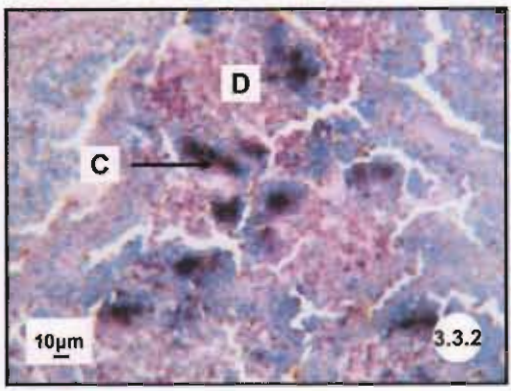

Figure 3.3.2: Acid phosphatase (red/brown) in the duct (D) and crypt cells (C) of the digestive gland of *H. discus hannai* (x400 digital camera).

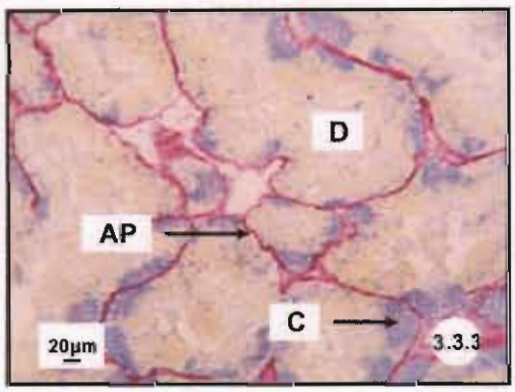

Figure 3.3.3: Alkaline phosphatase (AP) (pink) around the duct cells (D) and crypt cells (C) of the digestive gland of *H. discus hannai* (x200 digital camera).

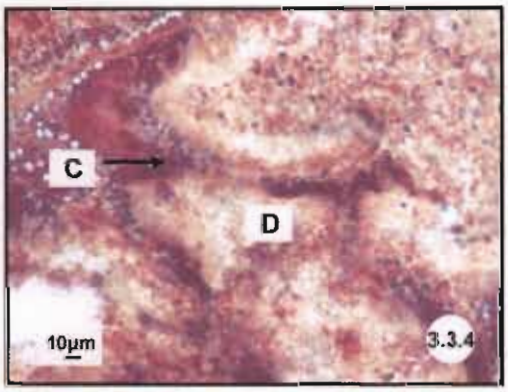

Figure 3.3.4: Alpha naphthyl butyrate esterase (brown/red) positive duct cells (D) and crypt cells (C) in the digestive gland of *H. discus hannai* (x400 digital camera).

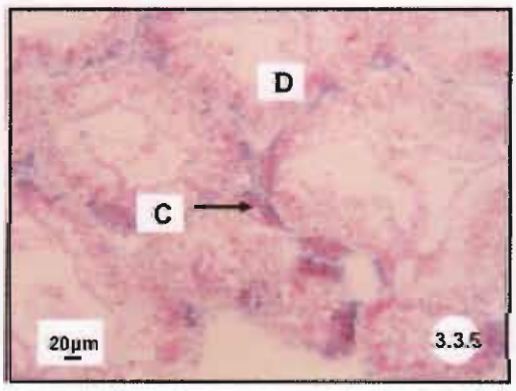

Figure 3.3.5: Chloroacetate esterase (pink) enzyme localised in the duct (D) and crypt (C) cells of the digestive gland of *H. discus hannai* (x200 digital camera).

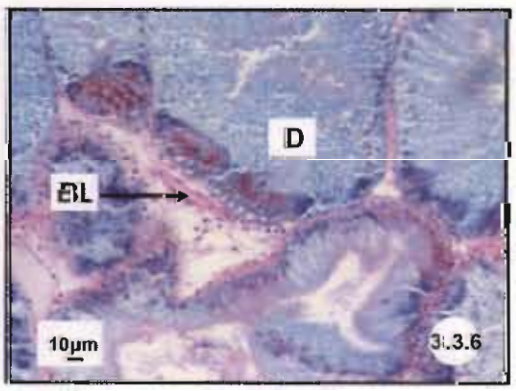

Figure 3.3.6: ABPAS: AB (blue) positive duct cells (D) and PAS positive (magenta) basement lamina (BL) in the digestive gland of *H. discus hannai* (x400 digital camera).

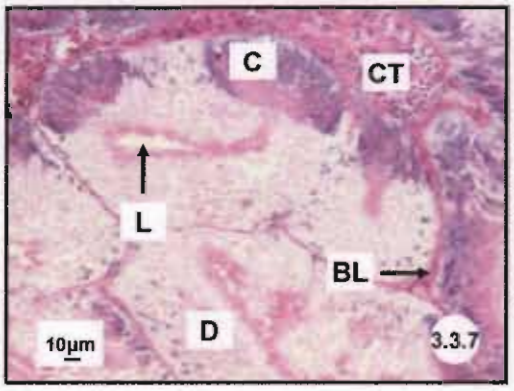

Figure 3.3.7: Periodic Acid Schiff: PAS positive (magenta) duct cells (D), crypt cells (C), lumen (L), connective tissue (CT) and basal lamina (BL) in the digestive gland of *H. discus hannai* (x400 digital camera).

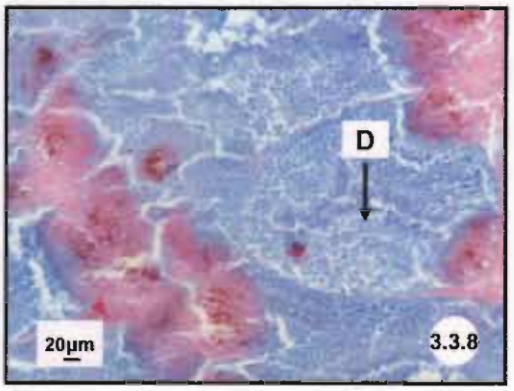

Figure 3.3.8: Alcian blue (pH 2.5): AB (blue) positive duct cells (D) in the digestive gland of *H. discus hannai* (x200 digital camera).

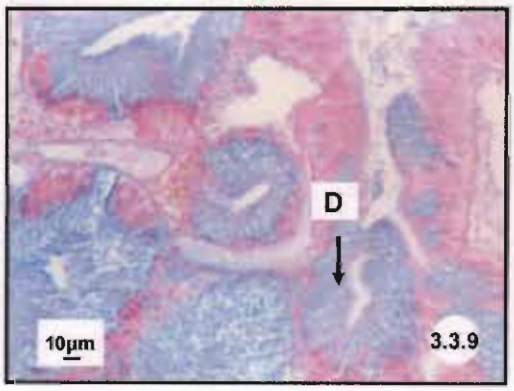

Figure 3.3.9: Alcian blue (pH0.2): strongly acidic mucins (blue) in duct cells (D) in the digestive gland of *H. discus hannai* (x200 digital camera).

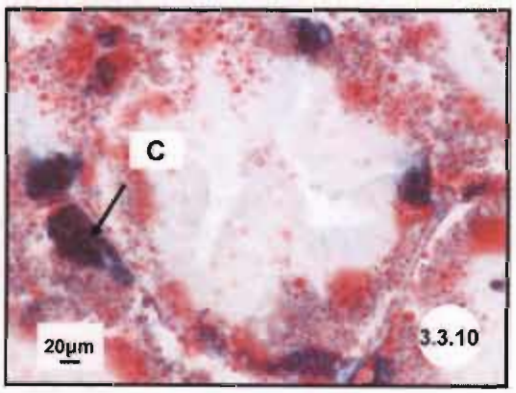

Figure 3.3.10: Lipid rich (red) crypt cells (C) in the digestive gland of *H. tuberculata* (x400 manual camera).

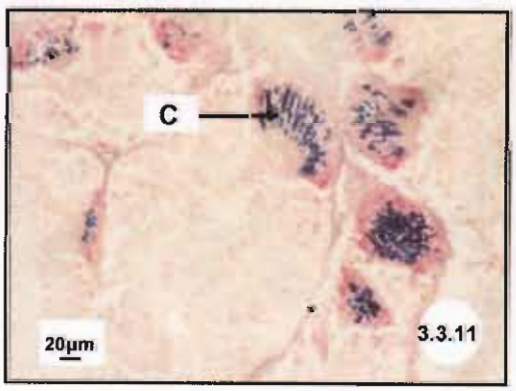

Figure. 3.3.11: Perl's Prussian Blue: positive iron granules (blue) in the crypt cells in the digestive gland of *H. discus hannai* (x400 manual camera).

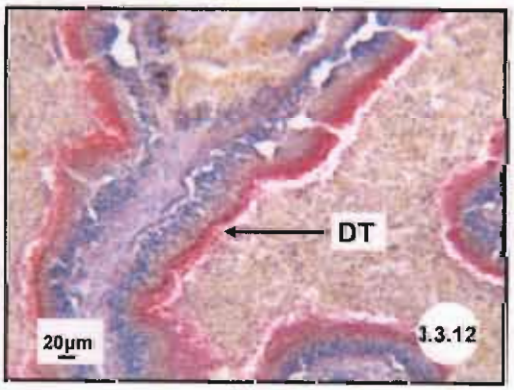

Figure 3.3.12: Leucineaminopeptidase (red) in the digestive tract (DT) of *H. discus hannai* (x200 digital camera).

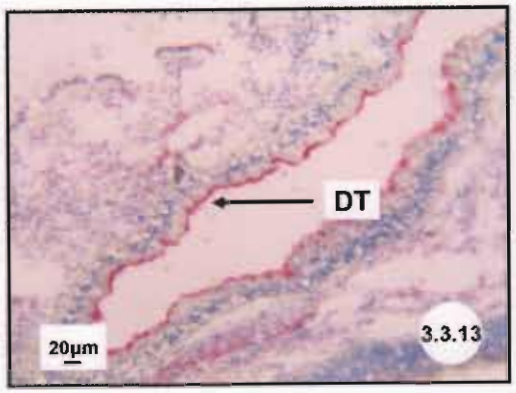

Figure 3.3.13: Alkaline phosphatase (pink) in the digestive tract (DT) of *H. discus hannai* (x200 digital camera).

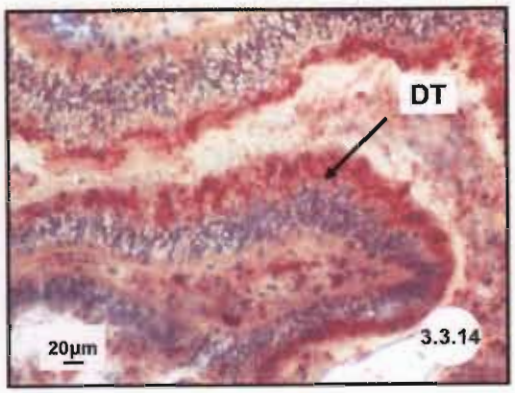

Figure 3.3.14: Alpha naphthyl butyrate esterase (red) in the digestive tract (DT) of *H. discus hannai* (x400 manual camera).

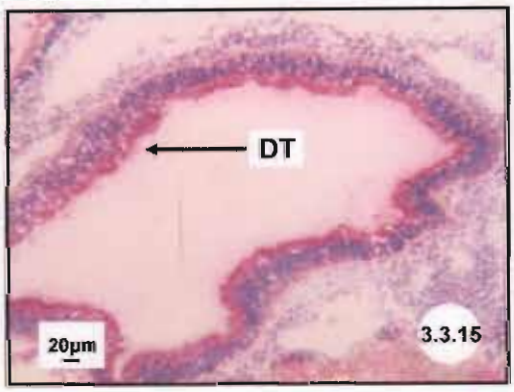

Figure 3.3.15: Chloroacetate esterase (pink) in the digestive tract (DT) of *H. discus hannai* (x200 digital camera).

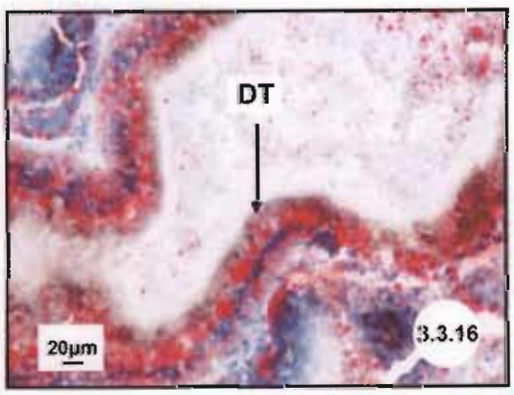

Figure 3.3.16: Oil Red O: Neutral lipids (red) in the digestive tract (DT) of *H. tuberculata* (x400 manual camera).

3.3.3 (d) Summary of Histochemistry

The secretory cells of the digestive gland expressed high levels of acid phosphatase, alkaline phosphatase, esterases, peroxidase and leucineaminopeptidase. Carbonic anhydrase and acetylcholinesterase were not expressed. The digestive gland was rich in carbohydrates: neutral mucins, acid mucins and strongly sulphated mucins. The secretory cells of the hepatopancreas were rich in lipids. The digestive gland was highly pigmented when stained with H&E. The granules were identified as iron. The cells of the digestive gland were negative for melanin, copper and calcium.

3.3.4 GILL

The paired gills/ctenidia of abalone are located along the sides of the mantle cavity. The gills are primarily composed of long filaments or lamellae (Crofts 1929). The lamellae arise from a central axis and are separated by a sparse connective tissue layer beneath the epithelium (Bevelander 1988). At the base of each filament are V-shaped skeletal rods that form a basal support and keep the filaments erect (Chitramvong et al. 2002) (Fig. 3.4.1). The epithelium is columnar and cuboidal. Squamous epithelium is sometimes found in the convoluted areas of the ctenidia. The cells in the basal regions of the lamellae are usually ciliated. Non-ciliated cells along the length of the gill filaments are interspersed with goblet cells, mucous cells and other gland cells.

3.3.4 (a) Epithelium of Gill Filaments

Enzymes

Acid and alkaline phosphatases were detected in similar areas of the gill tissue. Acid phosphatase was identified in the epithelia of the gill lamellae between the skeletal rods (Fig. 3.4.2). Weak alkaline phosphatase was detected in the epithelia between the upper regions of the skeletal rods (Fig. 3.4.3). Focal positivity was observed for esterases along the epithelia of the gill filament. The pattern of staining was similar for both enzymes (Fig. 3.4.4, 3.4.5). The gill epithelium was also positive for peroxidase (Fig. 3.4.6).

Carbohydrates

ABPAS revealed a strong AB positive area around the ctenidial axis indicating the presence of acid mucins (Fig. 3.4.7). The epithelium of the gill contained mucous cells that were PAS positive (Fig. 3.4.8).

The gill tissues exhibited strong alcian blue staining for acid mucins at both pH 2.5 and pH 0.2. Alcian blue at pH 2.5 stained acidic mucopolysaccharides intensely blue (Fig.

3.4.9) and at pH 0.2 strongly sulphated acid mucins stained positively in the mucous cells of the epithelium (Fig. 3.4.10).

3.3.4 (b) Skeletal rods

Carbohydrates

The skeletal rods were PAS positive for neutral mucin (Fig. 3.4.8) but were negative for acidic and strongly sulphated mucins.

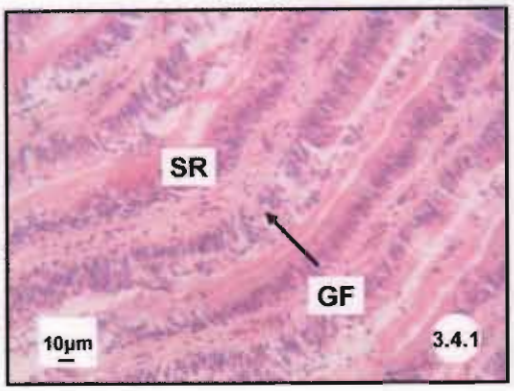

Figure. 3.4.1: H&E of abalone ctenidia showing gill filaments (GF) and skeletal rods (SR) of *Haliotis discus hannai* (x400 digital camera).

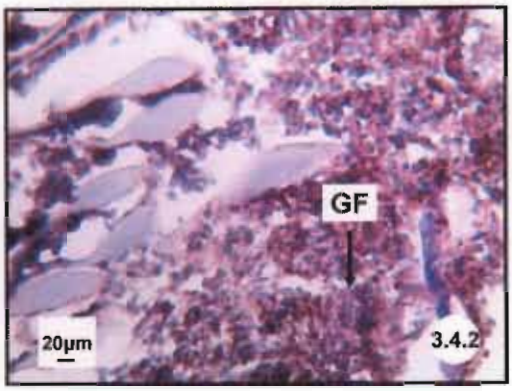

Figure 3.4.2: Acid phosphatase (red/brown) in the epithelia of the gill filaments (GF) of *Haliotis discus hannai* (x200 digital camera).

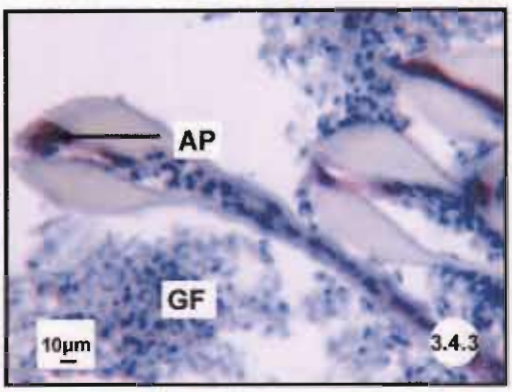

Figure 3.4.3: Alkaline phosphatase (AP) (pink) positive epithelia of the gill filament (GF) of *Haliotis discus hannai* (x400 digital camera).

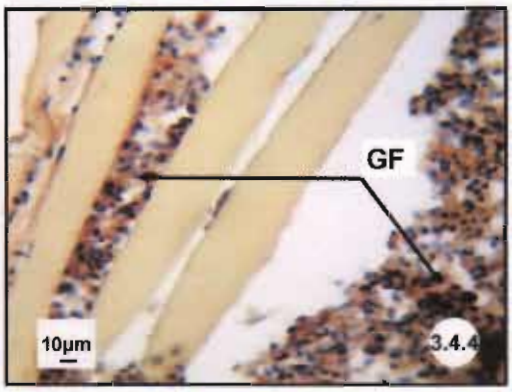

Figure 3.4.4: Alpha naphthyl butyrate (red/brown) esterase positive epithelium of the gill filaments (GF) of *Haliotis discus hannai* (x400 digital camera).

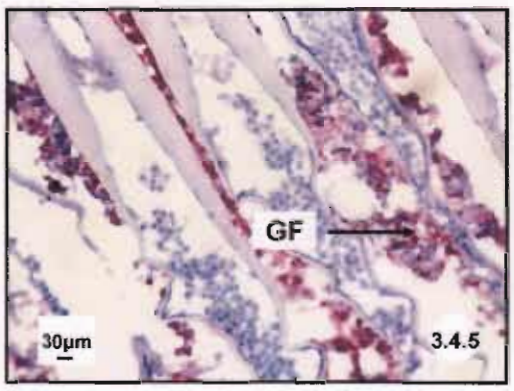

Figure 3.4.5: Chloroacetate esterase (pink) positive epithelium of the gill filaments (GF) of *Haliotis discus hannai* (x200 manual camera).

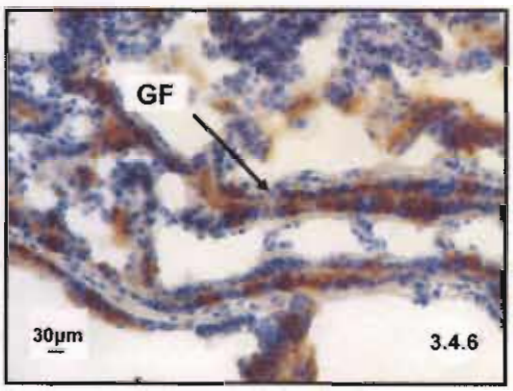

Figure 3.4.6: Peroxidase (brown) positive epithelia of the gill filaments (GF) of *Haliotis tuberculata* (x200 manual camera).

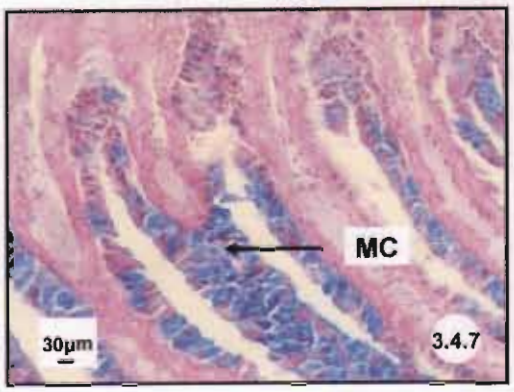

Figure 3.4.7: ABPAS: Acid mucins (blue) in the mucous cells (MC) of the gill epithelia of *Haliotis discus hannai* (x200 manual camera).

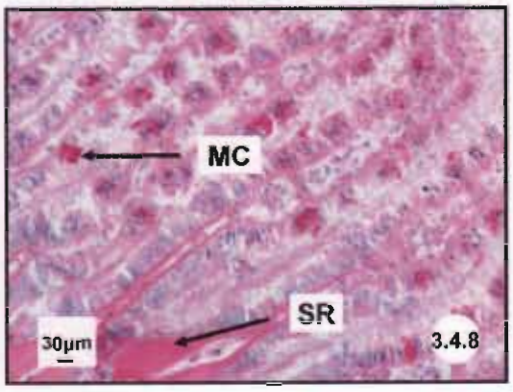

Figure 3.4.8: Periodic Acid Schiff: PAS positive (magenta) mucous cells (MC) in the gill epithelia; PAS positive skeletal rods (SR) of *Haliotis tuberculata* (x200 manual camera).

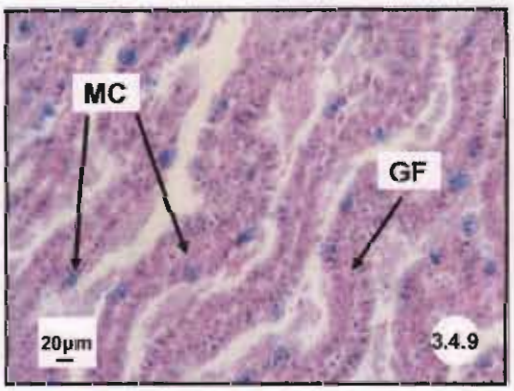

Figure 3.4.9: Alcian blue (pH 2.5): Acidic mucopolysaccharides (blue) in the mucous cells (MC) in the epithelia of the gill filaments (GF) of *Haliotis discus hannai* (x200 digital camera).

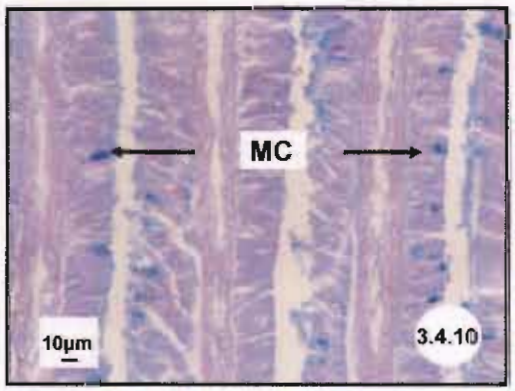

Figure 3.4.10: Alcian blue (pH 0.2): mucous cells (MC) containing strongly sulphated acid mucins (blue) in the gill epithelia of *Haliotis discus hannai* (x400 digital camera).

3.3.4 (c) Summary of Histochemistry

Histochemical studies of the gill epithelia and connective tissue detected a broad spectrum of enzyme distribution. Esterases, phosphatases, and peroxidase enzymes were expressed in the epithelium. Leucineaminopeptidase and carbonic anhydrase were not detected in the gill. The gill was positive for neutral mucin and acid mucin. Lipids and pigments were not detected in the cells of the gill. Enzymes, lipids and pigments were not detected within the skeletal rods.

3.3.5 HYPOBRANCHIAL GLAND

The hypobranchial gland is located in the mantle cavity of abalone along the side of the left gill and functions in the secretion of mucus, which clears debris from this region. It is composed of the following (Bevelander 1988):

1. Mucous cells that contain:
    a) Fibrous cytoplasm (acid mucopolysaccharides).
    b) Granular cytoplasm (glycoprotein).
2. Supporting ciliated cells.
3. Basal lamina.

The glandular epithelium of the hypobranchial gland is folded into large pleats that increase the secreting area (Fig. 3.5.1). Muscle and connective tissue join the inner surfaces of its folds.

3.3.5 (a) Mucous Cells:

Carbohydrates

Both types of mucous cells showed a similar pattern of staining for carbohydrates. Staining with combined ABPAS identified the presence of mainly acid mucins in the mucous cells (Fig. 3.5.2). The connective tissue in between the pleats or folds of epithelium was positive for both acid mucins and neutral mucins as the staining gave a combined colour of purple and blue. With PAS staining positive mucous cells were identified (Fig. 3.5.3). Acid mucins at pH 2.5 were identified in the mucous cells of the hypobranchial gland and the upper lining of the epithelial layer and the connective tissue between the pleats were also alcian blue positive (Fig. 3.5.4). Strongly sulphated mucins were present in the mucous cells (Fig. 3.5.5). The presence of N-acetyl sialomucin was not demonstrated using the mild PAS technique.

Pigments

The mucous cells of the hypobranchial gland were positive for copper. The copper granules were present towards the basal regions of the cells (Fig. 3.5.6).

3.3.5 (b) Basal Lamina

Carbohydrates

The outer lining of the basal lamina was strongly PAS positive (Fig. 3.5.3). The basal lamina was positive for acid mucins as verified by alcian blue staining at both pH 2.5 and pH 0.2 (Fig. 3.5.4, Fig. 3.5.5).

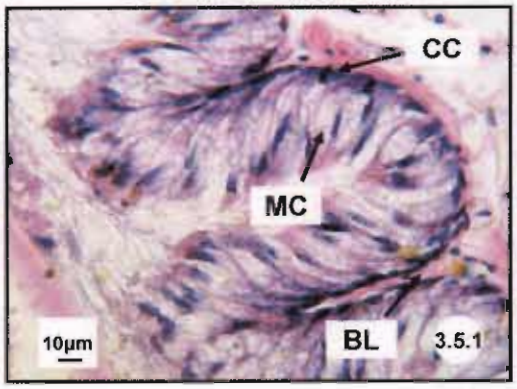

Figure 3.5.1: H&E of hypobranchial gland demonstrating mucous cells (MC), ciliated cells (CC) and the basal lamina (BL) of *Haliotis discus hannai* (x1000 oil manual camera).

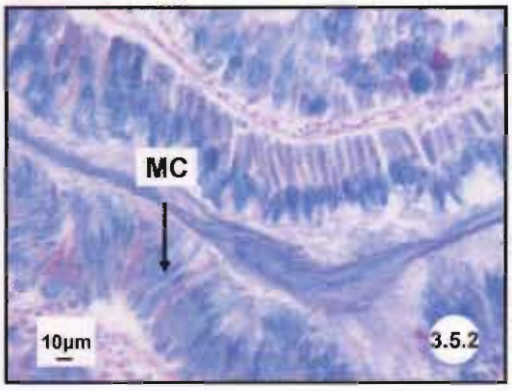

Figure 3.5.2: ABPAS: Acids mucins (blue) in mucous cells (MC) of the hypobranchial gland of *Haliotis discus hannai* (x400 digital camera).

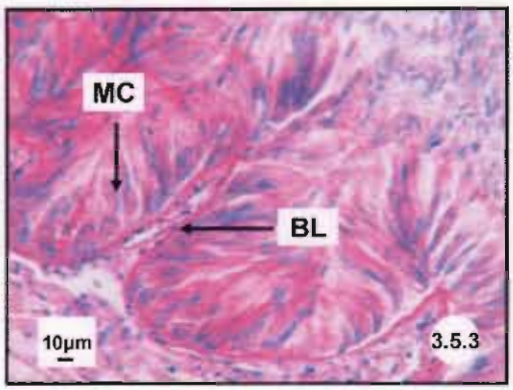

Figure 3.5.3: Periodic Acid Schiff: PAS positive (magenta) mucous cells (MC) and basal lamina (BL) in the hypobranchial gland of *Haliotis discus hannai* (x400 digital camera).

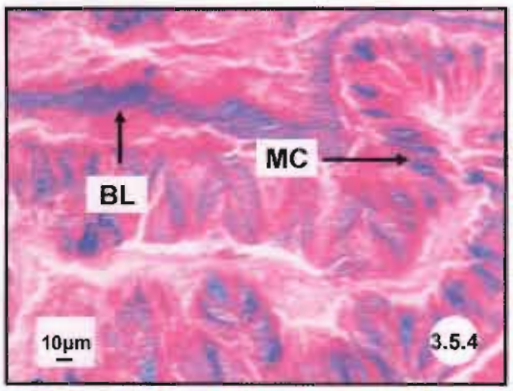

Figure 3.5.4: Alcian blue pH 2.5: Mucous cells (MC) containing acid mucins (blue) in the basal lamina (BL) of the hypobranchial gland of *Haliotis discus hannai* (x400 digital camera).

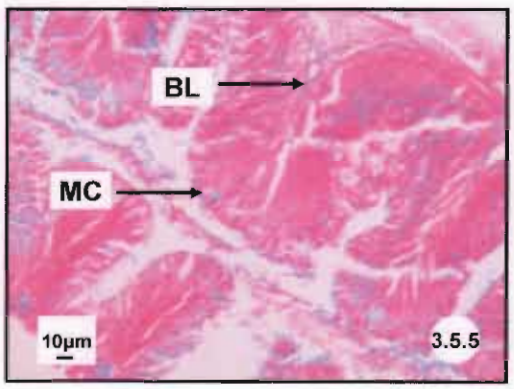

Figure 3.5.5: Alcian blue pH 0.2: strongly sulphated mucins (blue) in the mucous cells (MC) and basal lamina (BL) of the hypobranchial gland of *Haliotis discus hannai* (x400 digital camera).

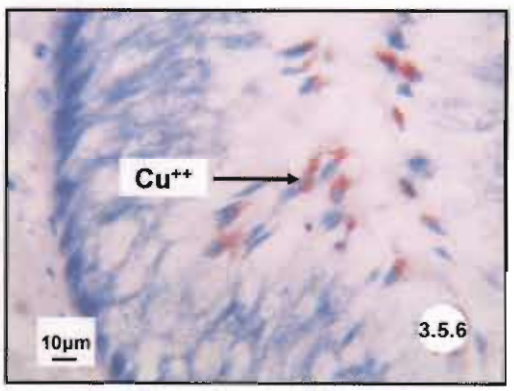

Figure 3.5.6: Rhodanine technique: Red copper granules (Cu^{++}) in the mucous cells of the hypobranchial gland of *Haliotis discus hannai* (x1000 oil manual camera).

3.3.5 (c) Summary of Histochemistry

The cells of the hypobranchial gland were rich in carbohydrates such as neutral, acidic and strongly sulphated acid mucins. Enzymes were negligible in the hypobranchial gland as was the presence of lipids. The hypobranchial gland of one *Haliotis discus hannai* sample was positive for copper granules. The ciliated cells of the hypobranchial gland are long slender dark staining cells that did not contain mucins or pigments.

3.3.6 GONAD

Abalone is a dioecious mollusc with a single gonad either male or female. It is a large racemose gland taking up a large part of the visceral mass. The epithelium and connective tissue grow out to form trabeculae. The epithelial cells grow out to produce either ova or sperm (Crofts 1929). The female ova consist of cytoplasm with an embedded nucleus. A vitelline layer encloses the cytoplasm and trabecular strands separate the ova (Fig. 3.6.1). The male sperm can only be seen under EM, as the gonad appears quite dense under LM (Fig. 3.6.2).

3.3.6 (a) Female Gonad

Carbohydrates

The trabecular strands were positive for acid mucins and the vitelline membrane contained mainly neutral mucin as indicated by combined ABPAS (Fig. 3.6.3). The trabecular strands enclosing the eggs and the vitelline membrane of the eggs were strongly PAS positive (Fig. 3.6.4). The connective tissue between the cells was also rich in carbohydrate. DPAS staining revealed weaker PAS staining of the vitelline membrane indicating that glycogen was present in addition to neutral mucin (Fig. 3.6.5). At pH 2.5 the trabecular strands of the eggs were alcian blue positive for acid mucin (Fig. 3.6.6) but the vitelline membrane was negative for acid mucin. At a lower pH of 0.2 the trabecular strands stained weakly for strongly sulphated acid mucin (Fig. 3.6.7).

Lipids

The contents of the eggs were full of lipid droplets. They were Oil red O positive for neutral lipids and phospholipids (Fig. 3.6.8).

3.3.6 (b) Male Gonad

Carbohydrates

The outer connective tissue of the male gonad was PAS positive consisting of neutral mucin and the gonadal ducts were also weakly positive (Fig. 3.6.9).

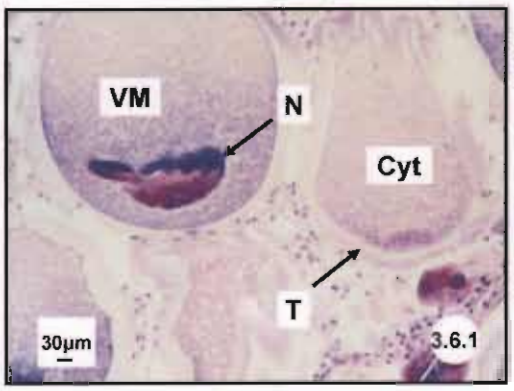

Figure 3.6.1: H&E of female gonad: Nucleus (N); trabeculae (T); cytoplasm (Cyt) and vitelline membrane (VM) of *Haliotis discus hannai* (x200 manual camera).

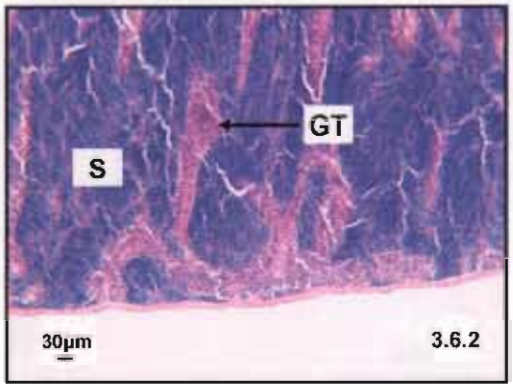

Figure 3.6.2: H&E of male gonad: Sperm (S) and gonadal tubules (GT) of *Haliotis tuberculata* (x200 manual camera).

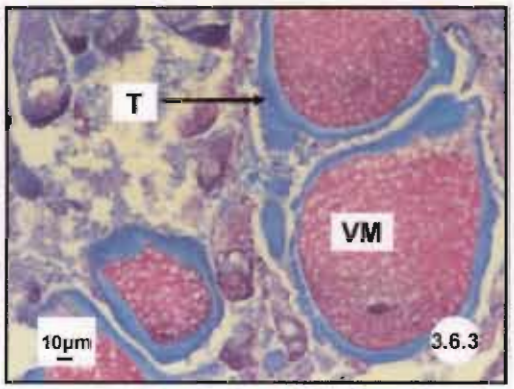

Figure 3.6.3: ABPAS of female gonad: PAS positive (magenta) vitelline membrane (VM) and AB (blue) positive trabeculae (T) of *Haliotis discus hannai* (x400 digital camera).

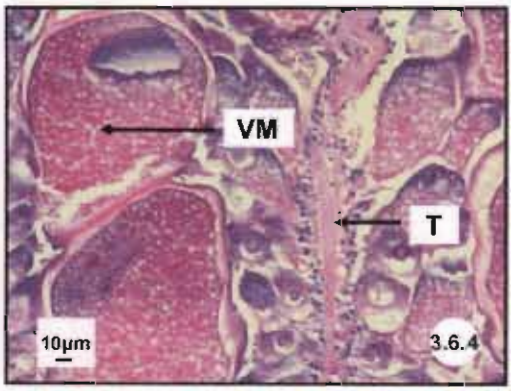

Figure 3.6.4: Periodic Acid Schiff: PAS positive (magenta) trabeculae (T) and vitelline membrane (VM) in the female gonad of *Haliotis discus hannai* (x400 digital camera).

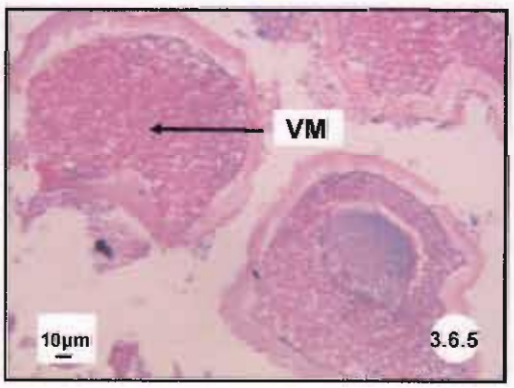

Figure 3.6.5: Diastase Periodic Acid Schiff (DPAS): Glycogen negative (magenta) vitelline membrane (VM) in the female gonad of *Haliotis discus hannai* (x400 digital camera).

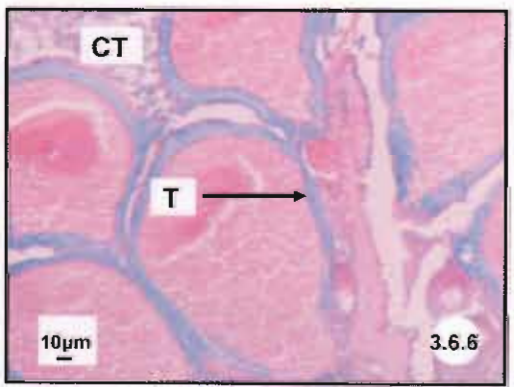

Figure 3.6.6: Alcian blue (pH 2.5); acid mucin (blue) in trabecular strands (T) and in the connective tissue (CT) of the female gonad of *Haliotis discus hannai* (x400 digital camera).

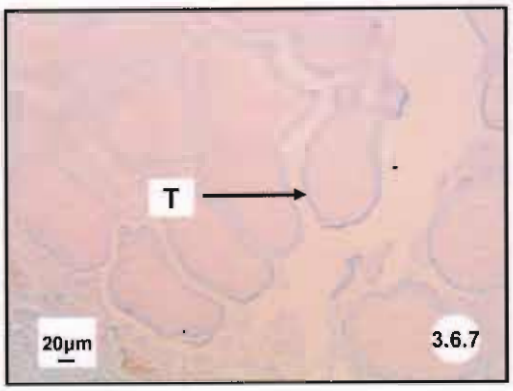

Figure 3.6.7: Alcian blue (pH 0.2): strongly sulphated acid mucins (blue) in the trabecular strands (T) of the female gonad of *Haliotis discus hannai* (x200 digital camera).

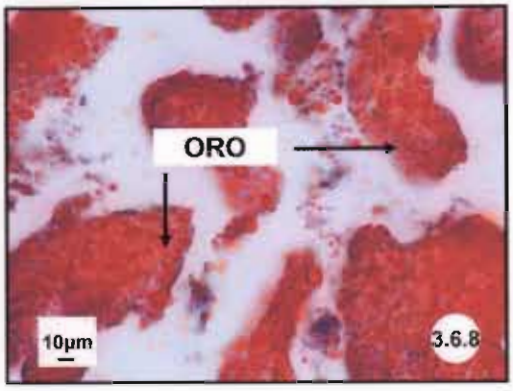

Figure 3.6.8: Oil Red O: Lipids (red) in the female gonad of *Haliotis discus hannai* (x400 digital camera).

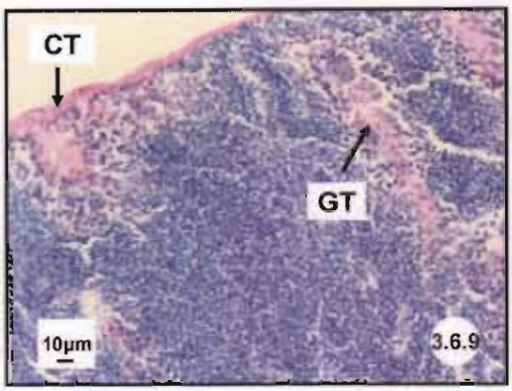

Figure 3.6.9: PAS positive (magenta) outer connective tissue (CT) and gonadal tubules (GT) of the male gonad of *Haliotis discus hannai* (x400 digital camera).

3.3.6 (c) Summary of Histochemistry

No enzymes were detected in the eggs of the female or in the sperm of the male abalone. The ova were rich in carbohydrates (neutral and acid mucin). The gonadal ducts of the male gonad were mainly positive for neutral mucins but no lipids or pigments were demonstrated. Carboxylated mucins and N-acetyl sialomucins were not present in either the female or male gonad.

3.3.7 KIDNEY

The kidneys are paired structures. The right kidney of abalone is made up of secretory epithelial cells that are squamous or cuboidal in shape with basally or centrally located nuclei (Bevelander 1988) (Fig. 3.7.1). These epithelial cells rest upon a well-defined basement membrane and surround a distinct lumen. The left kidney is very small (< 1 cm) and contains densely packed epithelial cells sitting on a connective tissue core. The cells are irregular cuboidal in shape and have prominent nuclei and granular inclusions (Chitramvong 2002) (Fig. 3.7.2).

3.3.7 (a) Renal Epithelia

Enzymes

Alkaline phosphatase was detected in the epithelial cells of the right renal organ (Fig. 3.7.3). The right renal epithelial cells exhibited non-specific esterases with strong expression of chloroacetate esterase activity (Fig. 3.7.4). Acid phosphatase and alpha naphthyl butyrate esterase were detected in the cuboidal epithelia of the right renal organ. The epithelial cells of the right renal organ were devoid of leucineaminopeptidase, peroxidase, acetylcholinesterase and carbonic anhydrase enzymes.

Carbohydrates

The basement membrane of the cuboidal epithelia of the right renal organ was weakly positive for neutral mucin (Fig. 3.7.5). Acid mucin was observed in the left renal organ (Fig. 3.7.6). The basement membrane of the cuboidal epithelium of the right renal organ was PAS positive for neutral mucin (Fig. 3.7.7). The luminal surface of the irregular epithelium of the left renal organ was PAS positive for neutral mucin (Fig. 3.7.8). Acid mucins were not detected in the right renal organ but the left renal organ was strongly positive for acid mucins (Fig. 3.7.9) and strongly sulphated acid mucins (Fig. 3.7.10).

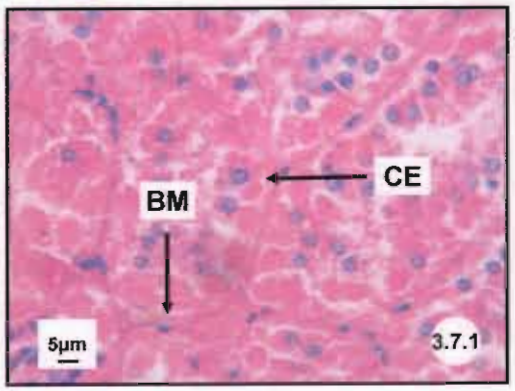

Figure 3.7.1: H&E of the right renal organ of *Haliotis discus hannai* showing cuboidal epithelia (CE) and underlying basement membrane (BM) (x1000 oil digital camera).

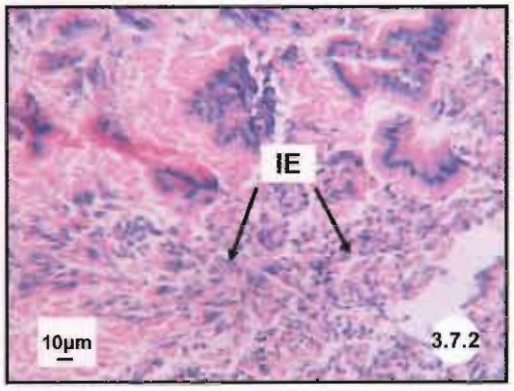

Figure 3.7.2: H&E of left renal organ of *Haliotis discus hannai* showing irregular epithelial cells (IE) (x400 digital camera).

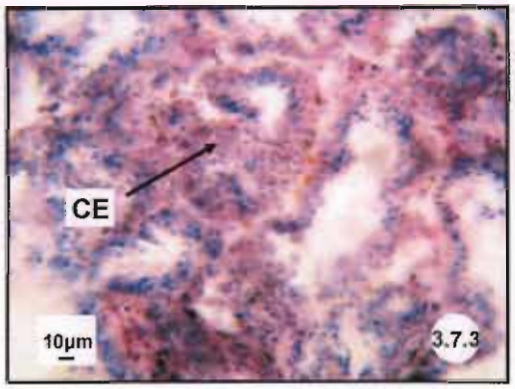

Figure 3.7.3: Alkaline phosphatase (pink) in the cuboidal epithelia (CE) of the right renal organ of *Haliotis tuberculata* (x400 digital camera).

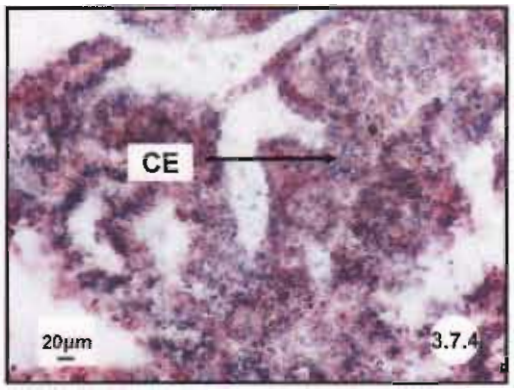

Figure 3.7.4: Chloroacetate esterase (pink) in cuboidal epithelial cells (CE) of the right renal organ of *Haliotis tuberculata* (x200 digital camera).

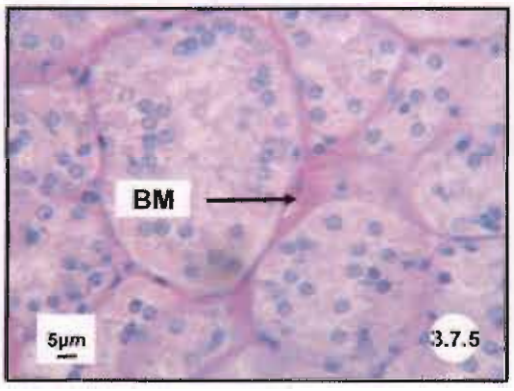

Figure 3.7.5: ABPAS: PAS positive (magenta) basement membrane (BM) of the right renal organ of *Haliotis discus hannai* (x1000 oil digital camera).

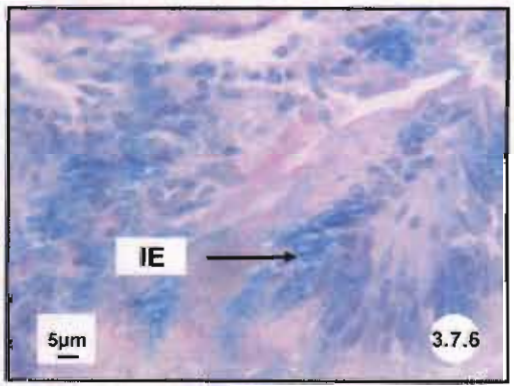

Figure 3.7.6: ABPAS: Acid mucins (blue) in the irregular epithelia (IE) of the left renal organ of *Haliotis discus hannai* (x1000 oil digital camera).

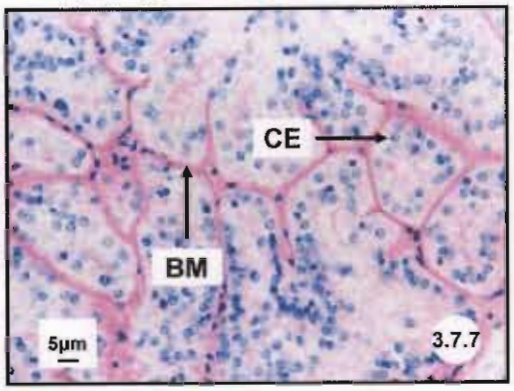

Figure 3.7.7: PAS positive (magenta) basement membrane (BM) of the cuboidal epithelia (CE) of *Haliotis discus hannai* (x1000 oil digital camera).

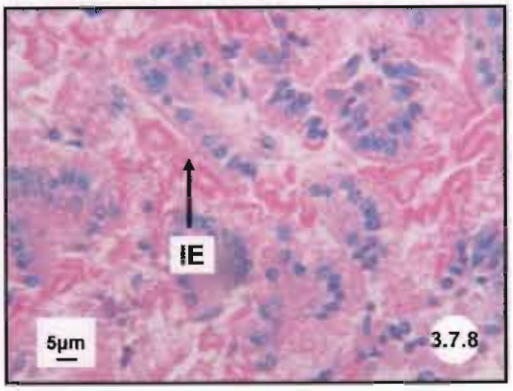

Figure 3.7.8: PAS positive (magenta) irregular epithelial cells (IE) in the left renal organ of *Haliotis discus hannai* (x1000 oil digital camera).

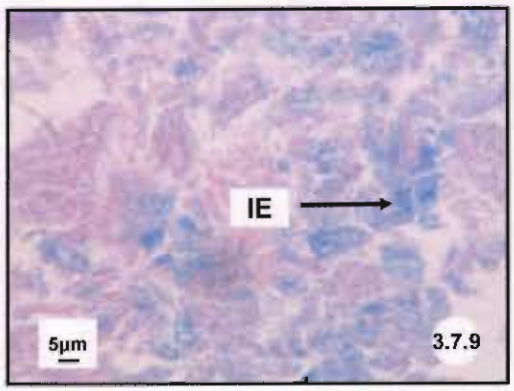

Figure 3.7.9: Alcian blue (pH 2.5): Acid mucins (blue) in the irregular epithelial cells (IE) of the left renal organ of *Haliotis discus hannai* (x1000 oil digital camera).

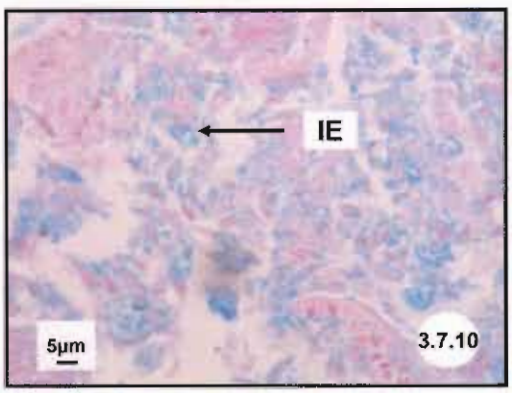

Figure 3.7.10: Alcian blue (pH 0.2): strongly sulphated mucins (blue) in irregular epithelial cells (IE) of the left renal organ of *Haliotis discus hannai* (x1000 oil digital camera).

3.3.7 (b) Summary of Histochemistry

The identification of enzymes in the kidney was confined to the right renal organ in this study as a result of insufficient samples of the left renal organ which was difficult to isolate due its small size. Both phosphatases and esterases were observed in the right renal organs of *Haliotis tuberculata* and *Haliotis discus hannai.* Acid mucins and strongly sulphated acid mucins were demonstrated in the epithelium of the left renal

organ. Acid mucins were not detected in the right renal organ. Lipids and pigments were not observed in the left and right renal organs.

OVERALL SUMMARY OF RESULTS

Tables 3.2-3.4 summarise the results for the histochemical analysis of enzyme, carbohydrate, lipid, pigment and mineral presence in abalone. Alkaline phosphatase and acid phosphatase were ubiquitously expressed in the epithelia of the foot, mantle, digestive gland and gill. Non-specific esterases were common to the same organs and appeared to have similar staining patterns. Acetylcholinesterase, a specific esterase, was detected in the foot and mantle but its activity was confined to the connective tissue. Leucineaminopeptidase was only detected in the epithelium of the digestive tract. Peroxidase was observed in the foot, gill and mantle epithelial tissues. Carbonic anhydrase was only observed in the foot epithelia.

Carbohydrates were expressed in almost all organs and tissues of abalone. Carbohydrate staining revealed neutral mucin, glycogen and acid mucin in the epithelial cells and connective tissue of the digestive gland, foot, gill, gonad, hypobranchial gland, renal organs and mantle. DPAS staining confirmed the presence of neutral mucin in most tissues. Acid mucins were the predominant mucin type found distributed throughout the organs of abalone with scattered expression of sulphated and carboxylated mucins.

Neutral lipids and phospholipids were confined to the digestive gland and gonad. Copper was located in the mucous cells of the hypobranchial gland and iron granules were identified in the secretory cells of the digestive gland. Calcium was expressed in the basement membrane of the foot and melanin was also located in the epithelial cells

of the foot. The same histochemical procedures were performed on both *Haliotis tuberculata* and *Haliotis discus hannai*. No apparent differences were found between these two species except for copper granules which were identified in the hypobranchial gland of one *H. discus hannai* sample and calcium which was identified in the basement membrane of the foot of one *H. discus hannai* sample.

Table 3.2

Summary of Histochemical Techniques and Enzyme Localisation

Target Enzyme	Organ of Enzyme Occurrence	Cell Location of Enzymes
Leukocyte acid phosphatase	Digestive system	Crypt & duct cells
	Foot	Columnar & cuboidal epithelia
	Gill	Columnar & cuboidal epithelia
	Mantle	Columnar epithelia
Acetylcholinesterase	Foot	Sub-epithelial ganglia
	Mantle	Connective tissue
Leukocyte alkaline phosphatase	Digestive system	Lining surface of crypt & duct cells; digestive tract epithelia
	Foot	Columnar & cuboidal epithelia
	Gill	Columnar & cuboidal epithelia
	Mantle	Columnar epithelia
	Right renal organ	Cuboidal epithelia
α-naphthyl butyrate esterase	Digestive system	Crypt cells & duct cells; digestive tract epithelia
	Foot	Cuboidal & columnar epithelia; sub-epithelial ganglia
	Gill	Columnar & cuboidal epithelia
	Mantle	Columnar epithelia; sub-epithelial ganglia; connective tissue
Hansson's Carbonic anhydrase	Foot	Columnar & cuboidal epithelia
Naphthol AS-D chloroacetate esterase	Digestive system	Crypt & duct cells; digestive tract epithelia
	Foot	Cuboidal & columnar epithelia; sub-epithelial ganglia
	Gill	Columnar & cuboidal epithelia
	Mantle	Columnar epithelia; connective tissue
	Right renal organ	Cuboidal epithelia
Leucineaminopeptidase	Digestive system	Lining digestive tract epithelia
DAB (diaminobenzidine)	Foot	Columnar & cuboidal epithelia
	Gill	Columnar & cuboidal epithelia
	Mantle	Columnar epithelia

Table 3.3

Summary of Histochemical Techniques and Carbohydrate Localisation

Staining Technique and Components Stained	Organ Location of Carbohydrates	Cell Location of Carbohydrates
Periodic Acid Schiff	Digestive system	Crypt cells; duct cells; connective tissue
Polysaccharides and carbohydrate-protein complexes	Foot	Columnar epithelia; sub-epithelial gland cells; muscle
	Gill	Columnar & cuboidal epithelia & skeletal rod
	Gonad	Female vitelline membrane & trabeculae; mal gonadal ducts; connective tissue
	Hypobranchial gland	Mucous cells; basal lamina
	Right renal organ	Basement membrane
	Left Renal organ	Irregular epithelia
	Mantle	Columnar epithelia; basement membrane; connective tissue
Alcian Blue/Periodic Acid Schiff	Digestive system	AB + duct cells; PAS + basal lamina
Acid and Neutral mucins	Foot	Columnar & cuboidal epithelia & sub-epithelial gland cells & ganglia
	Gill	Columnar & cuboidal epithelia
	Gonad	PAS + vitelline membrane; AB + trabeculae
	Hypobranchial gland	PAS + mucous cells
	Right renal organ	PAS + basement membrane
	Left renal organ	Irregular epithelia
	Mantle	AB + epithelia; PAS + basement membrane Columnar epithelia
Alcian Blue @ pH 2.5	Digestive system	Duct cells
Acid mucins	Foot	Columnar and cuboidal epithelia & sub-epithelial gland cells; connective tissue
	Gill	Columnar & cuboidal epithelia; connective tissue
	Gonad	Trabeculae
	Hypobranchial gland	Mucous cells; basal lamina; connective tissue
	Left renal organ	Irregular epithelia
	Mantle	Columnar epithelia
Alcian Blue @ pH 0.2	Digestive system	Duct cells
Sulphated Acid mucins	Foot	Columnar and cuboidal epithelia; sub-epithelial gland cells
	Gill	Columnar & cuboidal epithelia
	Gonad	Trabeculae
	Hypobranchial gland	Mucous cells; basal lamina
	Left renal organ	Irregular epithelia
	Mantle	Columnar epithelia
Methylation-Saponification technique	Foot	Sub-epithelial gland cells
Carboxylated mucins		

Table 3.4

Summary of Histochemical Techniques and Lipid, Pigment and Mineral Localisation

Staining Technique and Component Stained	Organ Location of Lipids, Pigments and Minerals	Cell Location of Lipids and Pigments
Lipids		
Oil Red O Neutral lipids and Phospholipids	Digestive system Gonad	Crypt cells; digestive tract epithelia Vitelline membrane
Pigments and Minerals		
Rhodanine Technique Copper	Hypobranchial gland	Mucous cells
Perl's Prussian blue Iron	Digestive system	Crypt cells
Masson Fontana Melanin	Foot	Cuboidal epithelia
Alizarin Red S Calcium	Foot	Basement membrane of columnar epithelia

3.4 DISCUSSION

3.4.1 Enzymes

The key aim of this study was to characterise some of the functional enzymes in abalone tissues using histochemical techniques. The presence of enzymes was assessed in two species of abalone: *Haliotis tuberculata* and *Haliotis discus hannai*. *Haliotis tuberculata* and *Haliotis discus hannai* exhibited similar profiles of enzyme distribution and cellular distribution of carbohydrates, lipids and pigments in this study. There have been many enzyme studies on shellfish concentrating on the structural and functional aspects of specific systems such as the digestive system or immune system. In this study all systems including the digestive, respiratory, excretory and reproductive systems are analysed for the presence of enzyme activity. This will contribute to the existing knowledge by creating a baseline of normal histology which is necessary for further studies of pathology. A detailed knowledge of the composition of these tissues will aid in understanding the functional activities ongoing within these tissues and alterations in such activites could be used as markers of disease. In total eight enzymes were detected in a range of abalone tissues using histochemistry on cryostat sections.

Phosphatases are ubiquitous enzymes in most organisms and can be specific or non-specific. Two phosphatases were examined in this study. Acid and alkaline phosphatases are a group of widely distributed enzymes that have a broad specificity and differ according to their source, but essentially they both act on a wide range of phosphate monoesters. Alkaline phosphatase functions in the hydrolysis of *S*-substituted monoesters while acid phosphatase hydrolyses the *O*-substituted monoesters of phosphorothioic acid (Dixon and Webb 1979). In this study acid and alkaline phosphatases were detected in all abalone epithelial tissues except the hypobranchial

gland and gonad. In the foot, acid and alkaline phosphatases were observed in the peripheral and pedal epithelia. Voltzow (1994) also reported alkaline phosphatase positive cells in the tubulous pedal glands of the winkle *Pomatias elegans*. In marine organisms, alkaline phosphatase is known to play an important role in cell phosphate metabolism, as it is involved in the absorption of phosphate and calcium from seawater and also in the process of biomineralisation (Xiao et al. 2002). Prosobranchs are also known to secrete a variety of proteins, polysaccharides, acids and salts from the foot (Voltzow 1994).

The columnar epithelia of the mantle also displayed focal staining of alkaline phosphatase. The mantle contains cells that excrete the precursors of the mineral and organic components of the shell (Bevelander 1988). According to Xiao et al. (2002) alkaline phosphatase is involved in this biomineralisation process.

Both columnar and cuboidal epithelia of the gills expressed acid and alkaline phosphatase in the study presented here. Both phosphatases were observed by Grizel et al. (2003) in the lateral cells of gill filaments in the oyster *Crassostrea gigas*. The gills function in blood transport, gaseous exchange and water movement (Bevelander 1988). Enzyme activity in the epithelial cells helps the ciliary cells to carry out the branchial functions (Grizel et al. 2003) and enzyme activity in hemocytes forms an important component of molluscan immune defence through hemocytic activity (Wootton and Pipe 2003).

Lysosomes are organelles containing hydrolytic enzymes which form an important element of the digestive and defence systems in adult molluscs (Wootton and Pipe

2003; Luna-González et al. 2004). Acid and alkaline phosphatases were detected in the cuboidal epithelia of the renal tubules in this study. This hydrolytic enzyme activity is most likely involved in the absorption of solutes from urine as the right kidney functions in nitrogenous excretion (Voltzow 1994). According to Voltzow (1994) secondary lysosomes are abundant in the excretory epithelia of the right and left renal organs.

Acid and alkaline phosphatases were assessed in the duct cells and crypt cells of the digestive gland and a similar pattern of enzyme distribution was observed in both cell types. Bevelander (1988) used Gomori's method to stain for alkaline phosphatase in the digestive system of abalone, which demonstrated the presence of this enzyme in the oesophagus. The results of the present study support the findings of Johnson et al. (1996) who used histoenzymology to detect acid phosphatase activity in the bivalve mollusc *Loripes lucinalis*. Strong acid phosphatase activity was observed throughout the digestive system with intense staining of epithelial tissue. These cells are involved in the absorption and intracellular digestion of food material confirmed by lysosomal activity in these regions (Johnson et al. 1996).

Esterases are enzymes that have an extremely low specificity and are often difficult to classify. Also, differences in specificity between corresponding enzymes from different species may arise. Simple esterases are involved in the hydrolysis of uncharged substrates and they can be categorised on the basis of aromatic ester hydrolysis (Dixon and Webb 1979). In this study we tested for the presence of two non-specific esterases in abalone tissues; α-naphthyl butyrate esterase and chloroacetate esterase. Chloroacetate esterase demonstrates the presence of enzymes in granulocytes using

naphthol AS-D chloroacetate as a substrate while alpha naphthyl butyrate esterase is an enzyme in monocytes and macrophages that uses alpha naphthyl butyrate as a substrate. Both esterase enzymes are non-specific and often produce similar patterns of staining.

In the pedal and peripheral foot epithelia both α-naphthyl butyrate esterase and chloroacetate esterase were located in the apex of the cells. These two enzymes were also detected in the mantle epithelia but produced a different pattern of reactivity, with α-naphthyl butyrate esterase confined to the apex of the cells and chloroacetate esterase activity present within the entire cell. Non-specific esterases in the mantle and foot have not been previously characterised in these shellfish. These esterases are most likely involved in shell secretion aiding carbonic anhydrase in the formation of the shell from the mantle. They could also be involved in locomotion through the breakdown of difficult substrata aiding in ease of movement as do mucins. Esterases also function as defence enzymes in hemocytes and could protect these shellfish against invading pathogens, through cytotoxic activities in the mantle and foot, which are two major organs in touch with the external environment. Esterase activity has been described in the gill epithelia and could be engaged in gaseous exchange, nutrient uptake or blood haematosis (Grizel et al. 2003). Phosphatase activity is known to be ubiquitous in shellfish gills but esterase activity has not been studied in great detail. In a study on the structural and functional characterisation of molluscan hemocytes, the activities of lysosomal enzymes including non-specific esterases were identified (Wootton and Pipe 2003). Non-specific enzyme levels in mussel hemocytes were analysed by Carballal et al. (1997) and are thought to form a component of the defence mechanisms of hemocytes. Thus the broad spectrum of activity of non-specific esterases is an indication of their multifunctional tasks.

Intense non-specific esterase activity was detected in the digestive system of abalone. Non-specific esterases are involved in the digestion of glycerol esters of fatty acids in most vertebrates including fish (Stevens and Hume 1995). Non-specific esterases have been detected in the epithelial cells of the intestine of the Nile tilapia, *Oreochromis niloticus* (Tengjaroenkul et al. 2002). Not many studies have focused on esterase characterisation in shellfish but some studies have looked at the effect of pesticides on enzyme activity, as the chemicals in pesticides are well known inhibitors of esterases (Mora et al. 1999; Galloway et al. 2002). Mora et al. (1999) investigated the two well-known pesticides, carbaryl and methylparathion in bivalves and concluded that molluscan cholinesterases are sensitive to these two pollutants rendering these bivalves sentinel speices in the monitoring of water pollution. The sensitivity of acetylcholinesterase and carboxylesterase enzymes to organophosphorous and carbamate compounds was studied by Galloway et al. (2002). It was observed that acetylcholinesterase inhibition was greater than carboxylesterase inhibition upon exposure to pesticides indicating its usefulness as an environmental biomarker. The use of esterases as biomarkers is a major area of research in environmental biology as it allows for pollution monitoring within marine habitats (Mora et al. 1999; Galloway et al. 2002; Bonacci et al. 2004).

Carbonic anhydrase has been reported in the foot of the winkle *Pomatius elegans* (Voltzow 1994) and in the mantle of *Collisella scabra* (rough limpet), and *Pinctada margaritifera* (black lipped pearl oyster) (Voltzow 1994; Grizel et al. 2003). Carbonic anhydrase intervenes in ion exchange, acid-base regulation and respiratory gas exchange (Sender et al. 1999). In the mantle this enzyme functions in the formation of the nacreous layer of the shell of the pearl oyster (*Pinctada margaritifera*) through the

process of mineralisation (Miyamoto et al. 1996). The present study confirmed carbonic anhydrase activity in the foot of *Haliotis discus hannai* and *Haliotis tuberculata* however this enzyme was not detected in the mantle of abalone. In the abalone foot, carbonic anhydrase activity is indicative of gaseous exchange. This is reinforced by Brown's (1984a) study of oxygen diffusion through the foot of the whelk *Bullia digitalis*. The absence of carbonic anhydrase activity in the mantle could be an indication of the transience of this enzyme in response to growth, reproduction or other seasonal activities.

Acetylcholine is a well-known neurotransmitter in the cholinergic systems of vertebrates and insects. Horiuchi et al. (2003) demonstrated the presence of acetylcholine synthesising activity in invertebrates including the abalone, *Haliotis discus*. Acetylcholine activity indicates the presence of cholinergic nerves in tissues. In this study acetylcholinesterase activity was detected in clumps of sub-epithelial ganglion cells in the foot and mantle of abalone. The foot and mantle are highly innervated organs of archaeogastropods. The mantle ganglia connect with the cerebral, pedal and posterior ganglia through the visceral loop. Talesa et al. (1995) identified two forms of cholinesterase in the gastropod *Helix pomatia*, a membrane bound form and a soluble form that catalyse the splitting of choline esters. As acetylcholine is a known neurotransmitter in molluscs (Muneoka and Twarog 1983; Talesa et al. 1998) the presence of acetylcholinesterase in the foot and mantle of abalone would be expected.

Peroxidases are widespread in aerobic cells using hydrogen peroxide as an oxidant. Classical peroxidases are haemoproteins (conjugated proteins in which the non-amino acid group or prosthetic group is heme or iron porphyrin) specific for peroxide using a

wide range of substances as donors (Dixon and Webb 1979; Lehninger 2005). In this study peroxidase was present in the epithelia of the foot, mantle and gill of abalone. A granular pattern of peroxidase staining was observed in the cells which are involved in the biochemical pathway in respiration as both the gill and mantle serve respiratory functions. Peroxidase forms many types of associations with microorganisms and is produced as a biochemical response to these associations (Small and McFall-Ngai 1999). Peroxidase has been identified in bivalve hemocytes and the release of oxygen metabolites from hemocytes has been associated with an internal defence system in molluscs (Wootton and Pipe 2003). The role of peroxidase in oxidative metabolism which is coupled to phagocytosis was also suggested by Carballal et al. (1997) when they measured enzyme levels in the hemocytes of the mussel *Mytilus galloprovincialis* and by Coles and Pipe (1994) who explored phenoloxidase activity in the hemolymph and hemocytes of the marine mussel *Mytilus edulis*.

Peptidases are proteolytic enzymes that hydrolyse peptide bonds in proteins. Aminopeptidases require a free amino terminus. Leucineaminopeptidase is an enzyme that has a high activity towards the peptides with an $_{L}$-leucine residue at the *N*-terminal end, with a free α–amino group. It acts on compounds with an *N*-terminal iso-leucine, valine, or aromatic amino acid and hydrolyses the amides of most amino acids (Dixon and Webb 1979). The results of this study show that this enzyme was confined to the epithelial cells of the digestive tract of both abalone species. Peptidases such as dipeptidyl-aminopeptidase (DAP II) and aminopeptidase (AMP I) have been reported in the oyster *O. edulis* (Grizel et al. 2003). More specifically leucineaminopeptidase activity in the alimentary tract of the abalone *Haliotis asinina* Linnaeus was recently demonstrated by Saitongdee et al. (2004) which supports results from our study. They

used an enzyme-histochemical method and according to their results activity of this enzyme is restricted to the brush border of the epithelial cells or in the lumen. Leucineaminopeptidase has been reported in the digestive system of *Polinices lewisii* (moon snail) and *Nassarius obsoletus* (eastern mud snail) and functions in digestion in the oesophagus (Voltzow 1994). Leucineaminopeptidase is also present in the columnar epithelial cells of the abalone intestine and has been linked to absorption (Saitongdee et al. 2004). The presence of leucineaminopeptidase in the intestinal epithelial cells is not solely confined to shellfish. Tengjaroenkul et al. (2002) reported the same results in the Nile tilapia (*Oreochromis niloticus*) using enzyme histochemistry and indicated its role in functional activities such as digestion and absorption.

3.4.2 Carbohydrates

Carbohydrates have been studied quite extensively in shellfish. The results of this study concur with published research on carbohydrates in that they are ubiquitous in shellfish tissues. They were identified in the tissues of the digestive gland, foot, gill, gonad, hypobranchial gland and mantle of abalone. The digestive gland was rich in polysaccharides and neutral mucopolysaccharides but also contained acid mucopolysaccharides (strongly sulphated mucins). The digestive gland of molluscs is a major carbohydrate store (Voltzow 1994), providing a major source of energy for these animals, and it has been previously documented that archaeogastropods store large amounts of glycogen in the digestive gland cells (Giese 1966; Voltzow 1994). There is some debate as regards the form of energy storage in abalone shellfish and whether it is lipid or carbohydrate. Lipid was described as the primary source of energy reserves for development in abalone by Moran and Manahan (2003) but according to Thongrod et al. (2003) high levels of lipid negatively affect abalone growth. Carbohydrates have

since been suggested as the major energy reserves in abalone (Du and Mai 2004) also worth noting is that abalone also have an immense capacity to synthesise lipid from carbohydrate stores (Durazo-Beltrán et al. 2003).

The hypobranchial gland is a mucus producing gland and the main components identified in this study were acid mucins, more specifically strongly sulphated acid mucin. These findings reinforce the role of this gland, as acid mucins would aid in the destruction of unfamiliar entities entering the mantle cavity, thus protecting the internal organs of the shellfish. A histochemical analysis of the hypobranchial gland of prosobranchs was published (Bolognani-Fantin and Ottaviani 1981) that identified the mucus produced by the mucous cells as strongly acidic, which is confirmed by the results of this present study.

The eggs in the gonad of the female abalone were identified as being very rich in carbohydrate, both acid mucin and polysaccharides. A study by Johnson et al. (1996) identified the gametes of the bivalve *Loripes lucinalis* as being extremely rich in carbohydrates and lipids prior to spawning which provides the larvae with energy in the veliger form. The vitelline membrane was rich in neutral mucin but negative for acid mucin while the outer trabecular strands were positive for strongly sulphated acid mucin.

Acid mucins were absent in the right renal organ but the left renal organ was positive for strongly sulphated acid mucins. This has not been previously reported; the left renal organ is actively involved in resorption and the lysis and assimilation of various components, which may explain the high incidence of acid mucins in this region

(Voltzow 1994). In the study presented here, the epithelial cells of the gill contained many mucous cells that contained predominantly acid mucin. These mucous cells were identified by Voltzow (1994) but the contents of these cells were not previously characterised.

The present study showed that the mucous cells in the foot contained acid and neutral mucins. These results corroborate previous studies that have indicated the role of these acidic mucopolysaccharides in locomotion (Grenon and Walker 1978). The mantle showed a similar pattern of carbohydrates in the epithelium with acid mucin being the major constituent. Acid mucopolysaccharides have previously been identified in the foot and mantle of various prosobranchs (Voltzow 1994). Carboxylated mucins were identified in the foot epithelia that have not been previously reported.

3.4.3 Lipids

The distribution of lipids in abalone tissues was demonstrated through lipid staining and the results did not differ considerably from the literature available on other shellfish species. Neutral lipids and phospholipids were demonstrated in two key organs; the digestive gland and gonad. It is well known that lipids are the most important energy source in animal tissues and are mostly stored as triacylglycerols (Durazo-Beltrán et al. 2003). Lipids in molluscan larvae have been used as an index for monitoring their physiological and nutritional status and potential for metamorphosis (Gallagher et al. 1986). Dietary lipids play important roles in providing concentrated energy, essential fatty acids and some other non-fat nutrients (Mai et al. 1995). The main storage component of the digestive gland of *Thais lamellosa* (frilled limpet) and most

archaeogastropods is lipid according to Voltzow (1994) which is supported by the observations made in this study.

Abalone meat is generally low in lipid which is similar to their natural diet of carbohydrate rich algae, however lipid can accumulate when abalone are fed a diet high in lipid (Mai et al. 1995; Dunstan et al. 1996). Most lipid is present in the cellular membranes and the viscera (Uki et al. 1986b; Dunstan et al. 1996). In this study this finding is reinforced as large quantities of lipid droplets were observed in the digestive diverticula.

Lipids have a tendency to accumulate to high levels in abalone gonads as they mature and contain a high level of n~3 HUFA (highly unsaturated fatty acid) indicating their role in reproduction and larval development of abalone (Pillsbury 1985; Delaunay et al. 1991; Robinson 1992). According to Moran and Manahan (2003) lipids are more important in fuelling the development of marine larvae than carbohydrate or protein as the lecithotrophic larvae are maintained on egg yolk at the veliger stage. Both lipid and protein are the dominant macromolecules that make up the biochemical composition of abalone. This has been confirmed in the present study as large quantities of lipid were localised in the female gonad of abalone by lipid histochemistry. Pioneering work on *Haliotis discus hannai* has linked dietary lipid quantity and quality to growth rates (Uki et al. 1985) rendering the supply of sufficient essential lipids important in the formation of successful artificial diets for abalone.

3.4.4 Pigments and Minerals

Both endogenous pigments and exogenous minerals are found in molluscs. In this study exogenous minerals were identified as iron in the digestive gland, copper in the hypobranchial gland and the endogenous mineral calcium was identified in the foot. Melanin is an endogenous pigment that was also identified in the foot. The digestive gland has a protective role in the sequestration of environmentally-derived heavy metals (Bryan et al. 1977; Carefoot et al. 2000) explaining the presence of iron granules within the crypt cells of the digestive gland or it could be a result of the algal rich diet which contains all essential nutrients and minerals. The hypobranchial gland functions in cleaning debris from within the mantle cavity, so it is possible that copper was acquired from the surrounding environment and the hypobranchial gland was attempting to flush this mineral out of the mantle housing. While copper is a micronutrient essential for life and is a component of haemocyanin, it can become toxic to many different cell types at high concentrations (Gaetke and Chow 2003; Hernández et al. 2006). These results offer considerable potential for a greater understanding of the functions of these organs. Iron and melanin as well as zinc and calcium stores (zinc was not identified in this study and calcium was only identified in the basement membrane of the foot) have been detected in prosobranch tissues (Bevelander 1988; Voltzow 1994). Melanin is a common pigment in many animal tissues and this study confined its presence to the epithelial cells of the foot.

Shellfish are used as environmental indicators because of their tendency to accumulate contaminants. The sedentary nature of shellfish means that they acquire metals and toxins from their surrounding environment quite easily. The quantity of metals in aquatic organisms may reflect the quantity of metals in the water. Most of the research

in this area is technology-based i.e. spectrophotometric analyses of trace metals in *Haliotis rubra* (Skinner et al. 2004) and biokinetic assessments of zinc accumulation in *Haliotis diversicolor supertexta* (Liao and Ling 1999; Liao et al. 2004). In the study presented here iron and copper were identified by histochemistry in abalone tissues. This has been reported in an Australian study on *Haliotis rubra* but these heavy metals posed no threat to their fisheries as their concentrations fell within the maximum permitted concentrations allowed by the Australia New Zealand Food Authority standards (Fabris et al. 2006). Iron and copper are metals that can be used as bioindicators, which indicates the potential of these easy, fast and low-cost histochemical methods in biomonitoring studies and environmental impact assessments. However it may also be the case that these metals are by-products of normal metabolism as a result of the presence of these metals in the substrates or diets of these shellfish, in which case metal concentration may fluctuate in response to seasonal and reproductive cycles.

Many cell components and cellular activities have been identified in this study generating valuable information in the form of an atlas of histochemistry, but further research on the structural and functional aspects of abalone will be important for understanding this species. Carbohydrates and lipids are often indicative of dietary intake which is a key component in the aquaculture industry so further research would be of significant value to shellfish farmers. Histochemistry has the potential to be used to demonstrate molecular changes within cells which can be important in pathological studies. Patterns of enzyme activity in shellfish often change in response to influences such as pathogens or pollution. As shellfish research has mainly focused on digestive enzymes, an extended panel of enzymes could be assessed within abalone tissues to

give a complete understanding of enzymatic activities in these shellfish. Further investigations into heavy metal accumulation could also be carried out, as aquatic organisms play a major role in pollution monitoring which is a vital element in maintaining environmental health and safety standards. The use of histochemical techniques generates significant information about the biology of this shellfish but also serves as a helpful adjunct in the study of shellfish pathology.

CHAPTER 4

MOLECULAR DETECTION OF *VIBRIO* SPECIES AND *PERKINSUS* SPECIES INFECTION IN ABALONE (*HALIOTIS* SPECIES).

4.1. INTRODUCTION

4.1.1 Disease in Shellfish Aquaculture

Aquaculture is an expanding, profitable and economically important activity worldwide. Infectious diseases represent the main challenge to the development of shellfish aquaculture, hence the need for increased vigilance against the spread of pathogens that have the potential to be detrimental to both wild and cultured shellfish stocks. Infectious disease is considered the chief limitation to production, in terms of quality, quantity, regularity and continuity (Mialhe et al.1995). The introduction of foreign pathogens to a shellfish culture system can be lethal for that population, as no previous resistance has been built up against exotic 'hitchhiker' organisms. Numerous catastrophes within the shellfish industry have been recorded over the years whereby the transplantation of shellfish has led to the inadvertent introduction of disease into aquaculture facilities (Margolis 1996). Not only have some diseases become a primary constraint to the growth and sustainability of the shellfish sector (OIE: Office International des Épizooties 2003) they have also led to the ruination of multibillion-dollar shellfish industries such as the demise of *Haliotis kamtschatkana* culture in British Colombia in 1980 due to the protistan *Labyrinthuloides haliotidis* (Bower 1987; Bower 2003). Commercial fisheries of the Portuguese oyster *Crassostrea angulata* in France in the 1970s were brought to the brink of collapse due to the spread of gill diseases. A rapid transition was subsequently made to the culture of the Pacific oyster *C. gigas* and the Portuguese oyster is now nearly extinct (Bower et al. 1994).

Monitoring shellfish populations for pathogens associated with disease principally depends upon traditional, time-consuming morphology-based diagnostic assays, and these pathogens are difficult to detect and recognise with these techniques, particularly

when they are present at low levels of infection (Bostock 2002). Numerous aquaculture projects have been, and are continually being, developed in poorer countries around the world, the one major drawback being that there is a severe lack of pathology facilities for the identification of disease outbreaks. As a result of this shortage, mortalities often arise that cannot be locally and quickly identified even in terms of infectious and non-infectious disease (Mialhe et al. 1995).

4.1.2 Pathogens of Abalone Shellfish

According to Bower (2000) abalone shellfish are susceptible to a number of diseases that are divided into three categories depending upon the severity of infection. Category one encompasses pathogens and diseases that have severe impacts and cause mortality amongst wild and cultured abalone such as *Vibrio fluvialis*, *Labyrinthuloides haliotidis*, *Perkinsus olseni*, sabellid polychaetes, withering foot syndrome and amyotrophia. Category two pathogens are usually benign in their hosts or have special life-cycle requirements that prevent their establishment in another location and include ciliates, kidney coccidia and nematode and trematode parasitism. However, the risk associated with these pathogens in naïve animals in new habitats is unpredictable. The third category includes ubiquitous non-pathogenic organisms that only become serious under adverse environmental conditions such as common fungi, *Vibrio* bacteria and shell boring organisms (Bower 2000). A number of new abalone pathogens (category one and two) have emerged since 2000 including numerous *Vibrio* species, haplosporidia, coccidia and rickettsia-like prokaryotes (Bower 2003).

The bacterium, *Vibrio harveyi* and the protozoan, *Perkinsus olseni* are two major pathogens of abalone shellfish. They have caused severe disease in abalone shellfish

throughout Europe and Australia (O'Donoghue et al. 1991; Goggin and Lester 1995; Nishimori et al. 1998; Nicolas et al. 2002) and have infected other shellfish species in Europe and Japan (Hamaguchi et al. 1998; Casas et al. 2002; Elston et al. 2004). The serious impact of these pathogens on shellfish culture facilities and the proximity of these disease outbreaks to Ireland, were among the selection criteria for the development of nucleic acid-based detection methods in this study.

4.1.3 *Vibrio* Pathogens of Abalone and other Shellfish

Diseases caused by *Vibrio* bacteria are responsible for major economic losses in aquaculture (Sparagano et al. 2002). The Vibrionaceae are a family of Gram-negative bacteria that are ubiquitous in aquatic environments and pose a serious threat for many species of shellfish (Thompson et al. 2004). *Vibrio* bacteria are widespread among molluscan shellfish as verified by Pereira et al. (2001) who identified various strains within 14 different species of shellfish in Portugal. Larval vibriosis of molluscs causes a systemic infection of larval shellfish tissues. The dissolution of muscle and connective tissue is caused by the production of an exotoxin resulting ultimately in the death of the larva (Bower et al. 1994). In adult shellfish different genera have different host impacts but in abalone the bacteria cause lesions to form on the external surface of the foot of the shellfish which spread into the visceral organs destroying the muscle and connective tissues (Bower 2000).

The last two decades have seen a significant rise in the taxonomic knowledge of the *Vibrio* genus. *Vibrio* was first discovered as *Vibrio cholerae* in humans by Filippo Pacini in 1854 (Thompson et al. 2004). While a few species are harmless there is a broad pathogenicity associated with the Vibrionaceae as a number of different species

infect a variety of host organisms. The following members of the Vibrionaceae frequently infect shellfish, both bivalves and univalves: *Vibrio anguillarum*, *Vibrio alginolyticus*, *Vibrio carchariae/harveyi*, *Vibrio fluvialis*, *Vibrio splendidus*, *Vibrio tapetis*, *Vibrio tubiashii* and *Vibrio vulnificus* (Bower et al. 1994; Thompson et al. 2004).

Molluscs that are susceptible to *Vibrio spp.* infection are mainly clams, oysters and abalone. *V. anguillarum* has arisen in juvenile oysters *Crassostrea gigas* and *Ostrea edulis* resulting in serious mortality (DiSalvo et al. 1978; Jeffries 1982). *Vibrio alginolyticus* and *V. splendidus* are identified pathogenic organisms of the clam *Ruditapes decussatus* and the abalone *Haliotis diversicolor* Lischke (Lacoste et al. 2001; Lee et al. 2001; Gómez-León et al. 2005) *V. harveyi* and *V. carchariae* which have been classified as the same species, are major pathogens of abalone (Nishimori et al. 1998; Lee et al. 2001; Nicolas et al. 2002; Bower 2003). *V. tapetis* or *Vibrio* P1 causes brown ring disease and is commonly encountered in the clams *R. decussatus* and *R. phillipinarum* (Castro et al. 1995; Romalde et al. 2002; Paillard et al. 2005). *V. tapetis* is also associated with nocardiosis in the oyster *C. gigas* (Friedman and Hedrick 1991; Friedman et al. 1991). *V. vulnificus* has been identified as a cause of mortality in clams (*C. virginica*), abalone (*H. discus hannai* INO) and oysters (Miceli et al. 1993; Coleman et al. 1996; Li et al. 1998; Harwood et al. 2004).

4.1.4 Diagnostic Techniques for *Vibrio* Species

Histology, electron microscopy and culture techniques are the most commonly employed methods for the detection and identification of *Vibrio* bacteria (Bower 2000). Nucleic acid-based techniques for the detection of members of the Vibrionaceae are

becoming more popular and have been used to detect *Vibrio* pathogens in environmental waters and in infected organisms, as they are more sensitive than traditional methods (Cunningham 2002; Campbell and Wright 2003; Conejero and Hedreyda 2003; Oakey et al. 2003). Many of the diagnostic techniques available are PCR-based methods and a number of multiplex PCR techniques are also available that allow for the identification of several species in one test (Kong et al. 1995; Lee et al. 2003; Gubala 2006).

The gene for bacterial 16S ribosomal RNA (16S rDNA) consists of highly conserved regions specific to species within the same genus which allows for their discrimination, hence its use as a genetic marker and identification tool (Wilk et al. 1995). Bacterial phylogeny has improved considerably in the last twenty years as a result of increased knowledge about these rRNAs. The 16S rRNA gene is used frequently in molecular assays for the detection and classification of *Vibrio* species (Dorsch et al. 1992; Kita-Tsukamoto et al. 1993; Marchesi et al. 1998; Sparagano et al. 2002).

Nucleic acid amplification (PCR) methods have been used to amplify DNA extracted from *Vibrio harveyi* bacterial strains which is a serious *Vibrio* pathogen associated with abalone disease. Oakey et al. (2003) targeted the 16S rRNA gene of *V. harveyi* and designed VH1/2 (*Vibrio harveyi* 1/2) primers to PCR amplify a 413 bp DNA fragment from this gene. The VH1 primer annealing site was identical for *V. harveyi* and *V. alginolyticus* while the VH2 primer showed a slight variation. This resulted in the amplification of DNA from the *V. alginolyticus* strain but apart from this the PCR is *V. harveyi* specific. A recent study by Conejero and Hedreyda (2003) isolated part of the toxR gene of *V. harveyi* and developed a PCR for the specific detection of this

bacterium. Oligonucleotides targeting *V. harveyi* genes were designed by Hernández and Olmos (2004) and incorporated into a PCR method for the species-specific identification of this pathogen.

Some studies have used PCR techniques for the classification and characterisation of *Vibrio harveyi* as opposed to detection (Pedersen et al. 1998; McDougald et al. 2000; Gomez-Gil et al. 2004). Multiplex PCR has also been developed for amplification of multiple bacterial strains of *Vibrio* species (Kong et al. 1995; Kong et al. 2002; Lee et al. 2003; Panicker et al. 2004).

While quantitative PCR has not been performed with *Vibrio harveyi* it has been used for the detection and enumeration of other *Vibrio* species such as *Vibrio vulnificus*, *Vibrio cholerae* and *Vibrio parahaemolyticus* (Blackstone et al. 2003; Campbell and Wright 2003; Harwood et al. 2004; Panicker et al. 2004; Takahashi et al. 2005; Gubala 2006). The majority of these assays employ SYBR Green I as the fluorogenic probe as it is sensitive and relatively inexpensive. It allows for the simple confirmation of amplicons by melt curve analysis which produces a melt temperature for an amplicon that corresponds to the detection of a specific sized fragment by gel electrophoresis (Giglio et al. 2003).

Pedersen et al. (1998) declared *Vibrio carchariae* a junior synonym of *Vibrio harveyi* as significant similarities were observed between these two species. In the present study a PCR method for the detection of DNA isolated from bacterial strains of *Vibrio harveyi* will be set up using primers designed by Oakey et al. (2003). This PCR will be used to detect *Vibrio harveyi* in alcohol fixed and paraffin embedded tissues of *Haliotis*

tuberculata. It will be modified into a multiplex PCR for the simultaneous amplification of the *V. harveyi* 16S rRNA gene and a housekeeping *Haliotis* spp. actin gene (actin primers will be designed using the Primer3 software (Whitehead Institute for Biomedical Research)). Multiplex PCR will allow for the determination of the integrity of DNA extracted from alcohol-fixed and paraffin embedded abalone tissues. A quantitative real-time SYBR Green PCR method will also be established for *Vibrio* measurement in abalone.

4.1.5 *Perkinsus* Pathogens of Abalone and other Shellfish

The first member of the genus *Perkinsus* to be described was *Perkinsus marinus* (Mackin et al. 1950). It was initially named *Dermocystidium marinum* rendering the syndrome 'Dermo Disease'. *Perkinsus* spp. infections are characterised by tissue destruction and loss of normal structure of affected organs. *Perkinsus* cells are usually encapsulated by the host's hemocytes resulting in the formation of yellow pustules or abscesses which cause the dissolution of the tissues (Villalba et al. 2004).

The members of this genus include eight different *Perkinsus* species currently described, while only six remain true *Perkinsus* members (Villalba et al. 2004). *P. marinus*, *P. olseni/atlanticus*, *P. qugwadi*, *P. chesapeaki*, *P. andrewsi* and *P. mediterraneus* are the six confirmed members of this genus (Villalba et al. 2004). Initially species were discovered and discriminated on the basis of morphology, host species and geographic location. Such methods of identification have been supplemented by nucleic acid-based techniques that have facilitated the classification of these organisms based on genetic characteristics (Villalba et al. 2004). *P. atlanticus* was originally described as a parasite of *Tapes decussatus* (Azevedo 1989) but molecular

analysis has revealed that *P. atlanticus* and *P. olseni* are actually conspecific (Murrell et al. 2002). A recent study by Burreson et al. (2005) revealed the synonymy of *P. chesapeaki* and *P. andrewsi* and it has also been concluded that *P. karlssoni* is not a true *Perkinsus* species.

Perkinsus pathogens infect a broad range of molluscs such as oysters, abalone, clams, scallops, cockles and mussels (Villalba et al. 2004). *Perkinsus atlanticus* has been associated with serious mortalities in the carpet shell clam *Ruditapes decussatus* and the manila clam *Venerupis philippinarum in* Portugal, Japan and Spain (Azevedo 1989; Hamaguchi et al. 1998; Casas et al. 2002; Elston et al. 2004). *Perkinsus olseni* is a severe pathogen of *Haliotis ruber* and *Haliotis laevigata* in Australian waters (Lester and Davis 1981; O'Donoghue et al. 1991; Goggin and Lester 1995; Jones and Creeper 2006). *Perkinsus marinus* is an identified protozoan pathogen of the eastern oyster *Crassostrea gigas* in North America (Audemard et al. 2004; Elandalloussi et al. 2004; Russell et al. 2004). Goggin et al. (1996) reported *Perkinsus karlssoni* in bay scallops in Canada.

4.1.6 Diagnostic Techniques for *Perkinsus* Species

To date *Perkinsus olseni* is the only *Perkinsus* species that has infected abalone shellfish and few nucleic acid-based techniques have been developed to detect this pathogen (Hamaguchi et al. 1998; De la Herrán et al. 2000; Casas et al. 2002; Audemard et al. 2004). Classic methods employed for *Perkinsus* spp. diagnosis are histology and incubation in Ray's Fluid Thioglycollate Medium (RFTM). It has been discovered that the optimal temperature range for *Perkinsus* infection (zoosporulation) is in the region of 19°C to 28°C (Casas et al. 2002), eliminating certain geographic regions whose

temperatures fall outside this range. However in light of this pathogen's ability to withstand low temperatures and to overwinter so that it can infect when conditions are favourable, it is necessary to have techniques available should an outbreak occur on importation of infected abalone from affected regions to Ireland, or on importation of opportunistic exotic pathogens to which Irish abalone have not been previously exposed to, increasing the likelihood of a disease outbreak.

Perkinsus species are now discriminated primarily with sequence data from the internal transcribed spacer region (ITS) and the non-transcribed spacer region (NTS) of the rRNA gene complex. The ITS regions of the eukaryotic ribosomal RNA gene complex are non-coding regions with high rates of evolution and are often used as targets in the identification of *Perkinsus* species (Goggin 1994; De la Herrán et al. 2000; Burreson et al. 2005). The intraspecific variation within the ITS region allows for easy discrimination among most *Perkinsus* species and can be used to analyse relationships within the genus. The ITS sequences have been used to resolve members of this species (Villalba et al. 2004). Many PCR diagnostic techniques have been developed based on this region of the ribosomal RNA gene complex.

PCR primers have been designed to specifically target the ITS region of the ribosomal RNA gene of all *Perkinsus* species excluding *Perkinsus qugwadi* (Casas et al. 2002). These primers were used to confirm that the aetiological agent of Perkinsosis in the clam *Tapes decussatus* from Spain was *Perkinsus atlanticus* (Casas et al. 2002). This PCR successfully detected *P. atlanticus* DNA in tissue samples of infected clam.

Hamaguchi et al. (1998) designed a PCR for the detection of a *Perkinsus* protozoan that was similar to both *Perkinsus atlanticus* and *Perkinsus olseni* in *Tapes philippinarum* from Japan. In their study primers were designed to produce a 455 bp amplicon from the ITS region and the PCR proved accurate, sensitive and also applicable to numerous samples of infected clam tissues. The ribosomal RNA gene region of *P. atlanticus* was characterised by De la Herrán et al. (2000) and a PCR-based diagnostic assay was developed to amplify a 554 bp DNA fragment from the IGS (intergenic spacer) region of ribosomal genes. The quantity of amplified product in their study positively correlated with the level of infection of *P. olseni* in clam samples.

The NTS region has not been incorporated into molecular assays as frequently as the ITS region due to the lack of information as regards the intraspecific variation within this region. The NTS and the small subunit of *Perkinsus atlanticus* were first described by Robledo et al. (2000). The interspecific variability of the NTS region prompted the development of a PCR to target this region which amplifies a 690 bp fragment of the NTS region of *P. atlanticus*.

Using the intergenic spacer (IGS) region domains of *Perkinsus atlanticus* and *Perkinsus marinus*, primers and probes were designed to produce a PCR-ELISA-based diagnostic test for the detection of both *Perkinsus* species (Elandalloussi et al. 2004). Samples were analysed using genus-specific primers, serial dilutions of *Perkinsus* DNA were amplified and visualised using Southern and ELISA assays. Both Southern and ELISA techniques were at least 100-fold more sensitive than visualisation of amplicons on ethidium bromide-stained agarose gels. The combined technique can measure DNA to levels as low as picograms and ELISA is less labour intensive and much faster (2 days)

than Southern analysis (4 days). The combination of PCR and ELISA increases the sensitivity of the diagnostic test that can be applied to numerous samples and can be easily adapted for the detection of multiple infections.

Quantitative real-time PCR is a novel area of research in molluscan disease. Very few studies have incorporated this technique into their research for the rapid detection and quantitation of disease. However the advantages of this advanced PCR method have been well elucidated in other areas of research. One paper that reports the use of real-time PCR for *Perkinsus* species detection is that by Audemard et al. (2004). In this study real-time PCR using SYBR Green I was developed to detect and quantify *Perkinsus marinus* in environmental waters. Comparisons of standard PCR with real-time PCR revealed that fewer copies of the ITS region DNA could be detected with real-time PCR, indicating that it confers greater sensitivity than standard PCR.

Perkinsus olseni and *Perkinsus atlanticus* are another set of synonymous species showing similarities between their DNA sequences (Murrell et al. 2002). In the present study a novel PCR method for the detection of *Perkinsus olseni*, a protozoan parasite of *Haliotis* spp. will be assessed. Primers targeting the ITS region of *P. olseni* will be designed using the Primer3 software (Whitehead Institute for Biomedical Research) and used in a standard PCR for the detection of *P. olseni* from pure cultures and *P. olseni* in *Ruditapes decussatus* paraffin-embedded samples. In addition, a quantitative real-time SYBR Green PCR for the detection and quantitation of *Perkinsus olseni* will be established.

4.2 MATERIALS AND METHODS

In sections 4.2.1-4.2.2, DNA extraction methods from all samples are outlined. In sections 4.2.3- 4.2.7, PCR amplification methods for *Vibrio harveyi* and *Perkinsus olseni* are optimised. In sections 4.2.8-4.216, the purification of PCR products required for producing cloned standards is described. Sections 4.2.17- 4.2.19, outline the quantitative PCR methods optimised for both *Vibrio harveyi* and *Perkinsus olseni*.

4.2.1 Bacterial and Protozoan Samples

Four isolates of *Vibrio harveyi* inoculated in marine agar slants were obtained from the Institut Francais de Recherche pour l'Exploitation de la Mer (Ifremer), France. Isolates of five other *Vibrio* species (Table 4.1) on cryogenic beads were obtained from the Marine Institute, Blanchardstown, Ireland. All *Vibrio* strains were cultured directly from the cryogenic beads and grown on marine agar (Difco^{TM} Unitech) at varying conditions (Table 4.1). Three samples of *Haliotis tuberculata* tissues infected with *Vibrio harveyi*, (diagnosed by IFREMER) preserved in alcohol, were also obtained from Ifremer. For paraffin embedded tissues a portion of each tissue sample was formalin-fixed (10% formalin), processed through various grades of alcohol and xylene and embedded in paraffin wax. For negative controls, *H. discus hannai* and *H. tuberculata* shellfish tissues from the Boet Mór Shellfish Farm, Clifden, Co. Galway, Ireland were analysed.

Five cultures of *Perkinsus olseni* in 1 ml ethanolic cell suspensions were received from Christopher Dungan in the Cooperative Oxford Laboratory, Maryland, USA. Paraffin embedded tissues of clam *Ruditapes decussatus* infected with *Perkinsus olseni* were obtained from the Olhão and Alvor coastal lagoons in Portugal via Maria Lyons-Alcantara of the Marine Institute, Blanchardstown, Dublin, Ireland.

Table 4.1 Bacterial Isolates and Growth Conditions

Species	Isolate Identification/ Reference Number	Optimal Growth Temperature	Microbiological Incubation Period
Vibrio harveyi (4 strains)	01/021 01/022 02/001 02/109	30°C	Overnight
Vibrio alginolyticus	NCIMB 1903	30°C	Overnight
Vibrio anguillarum	NCIMB 329	30°C	Overnight
Vibrio splendidus	NCIMB 2231	15°C	7 days
Vibrio tapetis	CECT 4600	15°C	7 days
Vibrio tubiashii	NCIMB 2164	30°C	Overnight

*All isolates were grown on marine agar supplied by Difco™ (Unitech, CityWest, Dublin).

4.2.2 Genomic DNA Extraction

Total genomic DNA was extracted from bacterial and protozoan cultures, tissue and paraffin embedded samples using the commercially available QIAamp® DNA Mini Kit (Qiagen Ltd, Crawley, UK). A different extraction protocol was used for the extraction of DNA from isolates, tissues and paraffin embedded tissues as the extraction criteria differ for each type of sample.

Bacteria (grown on marine agar from bacterial isolates) were removed from a culture plate with a sterile swab and suspended in 180 µl of lysis buffer ATL, samples were vortexed, 20 µl of Proteinase-K (QIAamp® DNA Mini Kit) was added and tubes were incubated at 56°C for 1-3 h. 200 µl of buffer AL was added and samples were incubated at 70°C for 10 min. 200 µl of ethanol was added, samples were transferred to QIAamp spin columns and centrifuged. 500 µl of buffer AW1 was added to the samples which

were centrifuged and the filtrate discarded. 500 µl of buffer AW2 was added and samples were centrifuged at full speed. Finally 200 µl of elution buffer AE was added and extracted DNA was stored at -20°C.

For alcohol-fixed tissue samples, 80 mg (at least 25 mg recommended) of tissue was cut up into small pieces to which 180 µl of buffer ATL was added with 20 µl of Proteinase-K and incubated at 56°C for 1-3 h with intermittent vortexing. After the Proteinase-K step the same protocol for extracting DNA from bacterial isolates was followed.

For paraffin embedded samples, 3-5 tissue sections of 15 µm thickness were placed in 1.5 ml centrifuge tubes, 1200 µl of xylene was added and tubes were vortexed. Tubes were centrifuged at full speed for 5 min at RT (15-25°C). After aspiration of the supernatant, 1200 µl of absolute ethanol was added, samples were centrifuged at full speed for 5 min and all ethanol was removed. This step was repeated and samples were incubated at 37°C for 10-15 min to allow ethanol evaporation. The tissue pellet was resuspended in 180 µl of buffer ATL with 20 µl of Proteinase-K, samples were vortexed and incubated at 56°C for 5 days. 20 µl of Proteinase-K was added to samples every 4-8 h. The same protocol for extracting DNA from bacterial isolates was followed after the Proteinase-K digestion step but was modified slightly reducing the final elution step to 50 µl of buffer AE from 200 µl. This step was repeated once to give a final volume of 100 µl. Extracted DNA was stored at -20°C.

Perkinsus olseni cells in ethanol were centrifuged at 3000 rpm (revolutions per minute). Pellets were washed twice in TE (Tris-EDTA) buffer (Appendix E) and resuspended in TE buffer. The protocol for DNA extraction from body fluids and blood was followed.

Briefly, 20 µl of Proteinase K was added to a 1.5 ml eppendorf tube and vortexed followed by 200 µl of sample. 200 µl of buffer AL was added to the tube, which was mixed by pulse vortexing for 15 sec. The sample was incubated at 56°C for 1 h. The tube was centrifuged briefly and 200 µl of ethanol was added to the sample, which was pulse-vortexed for 15 sec and centrifuged. The sample was transferred to the QIAamp Spin Column in a 2 ml collection tube and centrifuged at 8000 rpm for 1 min. The eluate was discarded and the column inserted into a new collection tube. 500 µl of buffer AW2 was added and the sample was centrifuged at 8000 rpm for 1 min. The collection tube containing the filtrate was discarded and the column was inserted into a new collection tube. 500 µl of buffer AW2 was added to the column and the tube was centrifuged at 14,000 rpm for 3 min. The column was inserted into a 1.5 ml eppendorf tube and 200 µl of buffer AE was added. The sample was incubated at RT for 1 min and centrifuged at 8000 rpm for 1 min. Extracted DNA was stored at –20°C.

4.2.3 PCR Method for *Vibrio harveyi* Amplification

Oligonucleotide primers that amplify a region of the 16S rRNA gene reported previously (Oakey *et al.* 2003) were used in this study for the amplification of DNA from *Vibrio harveyi* isolates (Fig. 4.1). Magnesium chloride titrations for optimal amplification were determined in this study (which differed from Oakey et al. 2003) and a concentration of 2.5 mM was selected for use. The PCR reaction was performed as follows: 1X PCR reaction buffer, 200 µM deoxyribonucleotide triphosphates (dNTPs), 100 ng/µl VH primers (VH-1 (5′-AAC GAG TTA TCT GAA CCT TC-3′) and VH-2 (5′-GCA GCT ATT AAC TAC ACT ACC-3′)), 2.5 mM magnesium chloride, 1 U Platinum® *Taq* polymerase (5 U/µl) (Invitrogen Ltd., Renfrew, UK), and 5 µl template DNA in a final reaction volume of 25 µl. Thermal cycling conditions with the VH-1 and

VH-2 primers were as follows: 94°C for 2 min, 30 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 2 min, and a final extension of 72°C for 5 min. The expected product size with this set of primers is 413 bp.

```
   1 caggcctaac acatgcaagt cgagcggaaa cgagttatct gaaccttcgg ggaacgataa
  61 cggcgtcgag cggcggacgg gtgagtaatg cctaggaaat tgccctgatg tgggggataa
 121 ccattggaaa cgatggctaa taccgcataa tgcctacggg ccaaagaggg ggaccttcgg
 181 gcctctcgcg tcaggatatg cctaggtggg attagctagt tggtgaggta agggctcacc
 241 aaggcgacga tccctagctg gtctgagagg atgatcagcc acactggaac tgagacacgg
 301 tccagactcc tacgggaggc agcagtgggg aatattgcac aatgggcgca agcctgatgc
 361 agccatgccg cgtgtgtgaa gaaggccttc gggttgtaaa gcactttcag tcgtgaggaa
 421 ggtagtgtag ttaatagctg cattatttga cgttagcgac agaagaagca ccggctaact
 481 ccgtgccagc agccgcggta atacggaggg tgcgagcgtt aatcggaatt actgggcgta
 541 aagcgcatgc ag
```

Figure 4.1. Nucleotide sequence of the 16S rRNA gene of *Vibrio harveyi*. The pink shaded sequence corresponds to the 413 bp sequence obtained by PCR amplification using the VH-1/2 primers (underlined) (NCBI *Entrez* Nucleotide search: AY264934).

4.2.4 Multiplex PCR Optimisation for *Vibrio harveyi* and *Haliotis* Species

A multiplex PCR was developed to detect *Vibrio* and abalone genes simultaneously in the same reaction using DNA extracted from alcohol fixed *Vibrio harveyi* infected *Haliotis tuberculata* tissues. Primers for the amplification of part of the abalone actin gene were designed in this study using the Primer3 software (Whitehead Institute for Biomedical Research) (Fig. 4.2). The PCR reaction mixture of 25 µl contained the following: 1X PCR reaction buffer, 200 µM dNTPs, 100 ng/µl VH primers, 100 ng/µl ACTIN primers (ACTIN-1 (5´-TCG GTA TGG GAC AGA AGG AC-3´) and ACTIN-2 (5´-CAC ACG GAG CTC GTT GTA GA -3´)) (Fig. 4.2), 2.5 mM magnesium chloride, 1 U Platinum® *Taq* polymerase and 5 µl DNA extract. Thermal cycler conditions for

the multiplex PCR were: 94°C for 2 min, 30 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 2 min, and a final extension of 72°C for 5 min. Amplification with the VH and ACTIN primers would produce a 413 bp and a 155 bp product respectively.

```
   1 ctagttgaac ttagtcagcg gccggcgaac tcgtgtgtga attttgtttg tgtttcaacg
  61 gaaaatttaa gtaatcaaaa tgtgtgacga agatgttgct gcgttggtta tcgacaacgg
 121 ctccggcatg tgtaaggccg ggttcgccgg tgatgacgcc cccagagccg tcttcccctc
 181 catcgtcggt cgtccccgtc atcagggtgt gatggtcggt atgggacaga aggacagcta
 241 cgtcggtgac gaggctcagt caaagagagg tatcctcacc ctcaagtacc ccatcgagca
 301 cggtatcgtc accaactggg acgacatgga gaagatctgg caacacacct tctacaacga
 361 gctccgtgtg gccccagagg agcaccctgt cctcctgact gaggctcccc tcaaccccaa
 421 ggccaaccgt gaaaagatga cccagatcat gttcgagacc ttcaactctc cagctatgta
 481 tgtagccatc caggctgttc tgtctctgta cgcttccggt cgtaccacgg gtattgttct
 541 ggactctggt gatggtgtca cccacactgt ccccatctac gagggttacg ccccttcccca
 601 cgccatcatg agactggatc ttgctggacg tgacctcaca gactacctca tgaagattct
 661 cactgagcgt ggttactcat tcaccaccac cgctgagaga gaaattgtca gggacatcaa
 721 ggagaaactc gactatgtag cactcgactt cgagcaggag atggctactg ctgcctcttc
 781 atcctccctg gagaagagct acgagcttcc cgacggtcag gtcatcacca tcggaaacga
 841 gagattccgt tgtccagagg ccctcttcca gccatccttc ttgggtatgg aatctgctgg
 901 tatccatgaa acaacataca actccatcat gaagtgcgat gttgatatcc gtaaagactt
 961 gtacgccaac actgttctct caggaggtac caccatgtac cccggcatcg ctgaccgtat
1021 gcagaaggag atcacagccc ttgcccccag cacaatgaag atcaaggtca tcgctcctcc
1081 agagaggaaa tactccgtct ggatcggagg ctccatcttg gcctctctgt caaccttcca
1141 gcagatgtgg atcagcaagc aggagtacga tgagtccggc ccatccattg tacacaggaa
1201 gtgcttctaa attgtaggac tcctgctagt attacgtacg gaacgttatg caaacaactt
1261 gtcatgtctg tgttcaaata cacacaacca aatatgttgg cgccatcgct caatttcgac
1321 ctgtgaacta tgctcaacag cccaaataag accctagcct gatacttgtg tgtctcgctg
1381 gctgtcatat cgatcaaact tgtacatgta ttcatctctg tgcaattggt catatccctt
1441 gttatgtcac ttttttgaag tttttttttat aaaaatgtta aaaattcatt cctgaataaa
1501 aaaaatagca aaaacataaa aaaaaaaaaa aa
```

Figure 4.2. Nucleotide sequence of the actin gene (complete cds) of *Haliotis spp.* The green shaded sequence corresponds to the 155 bp sequence obtained by PCR amplification using the ACTIN-1/2 primers (underlined) (NCBI *Entrez* Nucleotide search: AY380809).

4.2.5 Validation of *Vibrio* species PCR Amplification Specificity

The PCR using VH primers was applied to DNA from 4 isolates of *Vibrio harveyi* (Table 4.1), and 1 isolate of *Vibrio anguillarum*, *Vibrio alginolyticus*, *Vibrio splendidus*, *Vibrio tapetis* and *Vibrio tubiashii* to check for specificity. This PCR was repeated three times. The multiplex PCR was applied to DNA extracted from 3 *Vibrio harveyi* infected abalone fixed in alcohol and 3 *V. harveyi* infected abalone embedded in paraffin wax so as to establish the efficacy of the method.

4.2.6 PCR Method for *Perkinsus olseni* Amplification

A PCR method was developed for the detection of *Perkinsus olseni* DNA extracted from pure isolates and four *Ruditapes decussatus* paraffin-embedded samples. Primers for this PCR were designed using the Primer3 software (Whitehead Institute for Biomedical Research) (Fig. 4.3). An EcoR1 site was added to the 5′-end of both forward and reverse primers for cloning purposes (shown in orange text below). Magnesium chloride titrations for optimal PCR amplification were carried out in this study and a concentration of 3 mM was selected for use. The PCR reaction mixture of 25 µl contained the following: 1X PCR reaction buffer, 200 µM dNTPs, 100 ng/µl ITS primers (ITS-1 (5′- GAC TGA ATT CTC TGC GAA ACT AGC GGT CTT-3′) and ITS-2 (5′- ACG TGA ATT CTG CAA ATC GCA GTG CTT ATC -3′)), 3 mM magnesium chloride, 1 U Platinum® *Taq* polymerase and 5 µl DNA. Thermal cycling conditions for PCR with ITS primers were as follows: 95°C for 5 min, 35 cycles of 95°C for 30 sec, 61°C for 30 sec and 72°C for 30 sec, and a final extension of 72°C for 5 min. The expected product size with this set of primers is 245 bp and with the additional EcoR1 sites the total product size is 265 bp.

```
  1 acaccgattc attctctgcg aaactagcgg tcttgcttcg gcgagatggg atccccgctt
 61 tgtttggatc cccccacctg accgccttaa cgggccgtgt taggtgatta tctcctatga
121 accattgtac tagtcacagt atccaaatcc ttttggattt tggtatttca aaacgaaatt
181 ccaaactctc aacgatggat gcctcggctc gagaatcgat gaaggacgca gcgaagtgcg
241 ataagcactg cgatttgcag aattccgtga accagtagaa atctcaacgc atactgcaca
301 aaggggatct ttcctctttg tacatacata tcagtgtcgc tcttcttccc gatacaaaca
361 ttttgttgtt aacgcaactc agtgctttgt atcccgcttg gactaactct tcggagatag
421 ttcgttatgt gcgcttgtga aggcaggcgt attaatttgc aaggctataa tctcgtattg
481 tagcccctcc gagaggagga ccgcgcctgt gagtgtcttt ggatgctcgc aagtccgact
541 gtgttgtggt gatatcacgt gttccttgat cacgcgattc ttctcttcaa cgcattatgt
601 caattcttga tgaaatgcag agaagtgttt ggatcacgcg ttcagtctgg tcgcgagata
661 gctatatatc atagcacgct tgtcggtttg caccatggca aattgtcatc att
```

Figure 4.3. Nucleotide sequence of the ITS 1 of *Perkinsus olseni.* The blue shaded sequence corresponds to the 245 bp sequence obtained by PCR amplification using the ITS-1/2 primers (underlined) (NCBI *Entrez* Nucleotide search: AY820757). The additional EcoR1 site (shaded orange in Section 4.2.6) of 20 bases (not shown here) yields a product of 265 bp.

4.2.7 Visualisation of PCR Products

PCR reactions were carried out using a PTC-100 thermocycler. Amplicons were visualised using 2% agarose gel electrophoresis in 1X Ultrapure™ TBE (Tris Borate EDTA) buffer (Invitrogen Ltd, Renfrew, UK) containing 0.01 µg/µl ethidium bromide at 120V for 1 h.

4.2.8 Preparation of *Vibrio harveyi* and *Perkinsus olseni* PCR Products for Cloning and DNA Quantitation

The following steps (4.2.8 – 4.2.19) outline the procedures involved in cloning both the *Vibrio harveyi* and *Perkinsus olseni* PCR products. It was necessary to clone the PCR products and quantify DNA concentration by fluorometry to create standards of known

DNA concentration. The concentration of the unknown target sequence was extrapolated from the standard curve which was generated from the cloned standards.

4.2.9 Gel Purification of *Perkinsus olseni* PCR Product

Cloning of the *Perkinsus olseni* PCR product required a pre-cloning purification step. The QIAquick® Gel Extraction Kit (Qiagen, Crawley, UK) was used to purify the 265 bp amplicon from a 2% agarose gel. The DNA fragment was excised from the gel using a clean scalpel under UV light. The gel was weighed in an eppendorf tube and 3 volumes of buffer QG was added to 1 volume of gel sample. The sample was incubated at 50°C for 10 min with intermittent vortexing. When the gel dissolved completely, 1 gel volume of isopropanol was added to the sample and mixed. The sample was transferred to a QIAquick® spin column in a 2 ml collection tube which was centrifuged at 13,000 rpm for 1 min. The eluate was discarded; the column was replaced into the collection tube and 500 µl of buffer QG was added and centrifuged for 1 min at 13,000 rpm. 750 µl of buffer PE was added to the column, it was centrifuged for 1min at 13,000 rpm and left to stand at RT for 2-5 min. The eluate was discarded and the column was centrifuged at 13,000 for 1 min. The column was placed into a clean 1.5 ml eppendorf tube, 30 µl of buffer EB was added and the tube was allowed to stand for 1 min before being centrifuged at 13,000 rpm for 1 min. Purified DNA was stored at -20°C. The *Vibrio harveyi* product cloned directly using the kit and did not require gel purification.

4.2.10 Cloning of *Vibrio harveyi* and *Perkinsus olseni* PCR Products

Vibrio harveyi and *Perkinsus olseni* PCR products were cloned into the pCR®2.1-TOPO® plasmid vector (Fig. 4.4) using the TOPO TA Cloning® kit (Invitrogen Ltd.,

Renfrew, UK). The TOPO cloning reaction consisted of 2 µl of fresh PCR product, 1µl of salt solution, 2 µl of sterile water and 1 µl of TOPO Vector (3.9 kb). The reaction was incubated at RT for 5 min, and then incubated on ice.

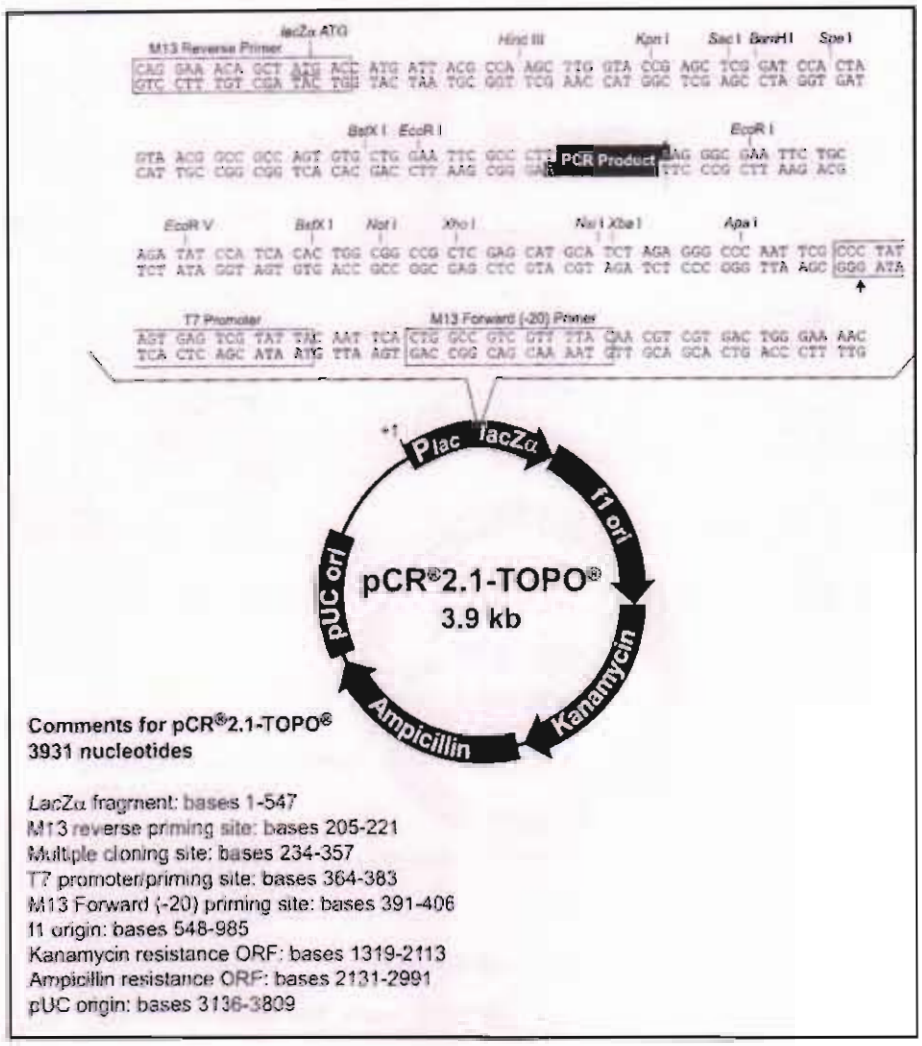

Figure 4.4. Diagram of pCR2.1®-TOPO vector and the sequence surrounding the TOPO® Cloning site (Taken From: TOPO TA Cloning® Manual).

4.2.11 Transformation of One Shot TOPO 10 Competent Cells

Chemically competent *Escherichia coli* cells supplied with the TOPO TA Cloning® kit (Invitrogen Ltd., Renfrew, UK) were thawed at RT and 6 µl of the TOPO cloning reaction was added to the cells, which were incubated on ice for 30 min. Cells were heat

shocked at 42°C for 30 sec, immediately transferred to ice and 250 µl of SOC (Super Optimal Catabolite Repression) medium (Appendix E) was added. Tubes were placed on a horizontal shaker at 200 rpm at 37°C for 1 h. After 1 h, 10-50 µl of each transformation was spread onto selective LB (Luria-Bertani) agar plates (Appendix E) containing 50 µg/ml ampicillin and 40 µl of 40 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) in DMF (N,N-dimethyl formamide) (Appendix E). Plates were incubated overnight at 37°C.

4.2.12 Colony Selection and Growth

10 colonies from each plate were chosen and placed in 4 ml of LB broth (Appendix E) containing 50 µg/ml ampicillin. Broths were incubated overnight at 37°C for 16-18 h.

4.2.13 Miniprep of Cultures for Insert Identification

A rough miniprep of cultures was carried out to purify the plasmid DNA and verify that the insert was present. 1.5 ml of overnight culture was placed into an eppendorf tube and centrifuged @ 13,000 rpm for 3 min at 4°C. The supernatant was removed and resuspended in 100 µl of ice-cold solution I (Appendix E). Samples were vortexed vigorously, 200 µl of freshly prepared solution II (Appendix E) was added and incubated on ice for 5 min. 150 µl of ice cold solution III (Appendix E) was added, samples were vortexed and incubated on ice for 5 min. Samples were centrifuged at 4°C for 5 min. The supernatant was transferred to a new tube and equal volumes (400 µl) of phenol/chloroform (Phenol:Chloroform:Isoamyl Alcohol 25:24:1) were added, samples were vortexed and centrifuged at 13,000 rpm for 5 min. The supernatant was transferred to a new tube and 800 µl of 100% ethanol was added. Samples were mixed, incubated on ice for 5 min and centrifuged at 13,000 rpm at 4°C for 5 min. The supernatant was

aspirated and 1 ml of cold 70% ethanol was added. Samples were centrifuged at 13,000 rpm for 5 min. The supernatant was aspirated and the pellet was allowed to air dry completely. Pellets were redissolved in 25 µl of TE buffer containing 22 µg/ml RNaseA, vortexed and left at RT for at least 15 min. Samples were stored at -20°C.

4.2.14 Restriction Digest of Minipreps

A restriction digest was performed on samples to cut the insert out of the plasmid and verify its presence in the plasmid. 10 µl of purified DNA, 7 µl of double distilled water, 2 µl of EcoR I buffer and 1 µl of EcoR I (20,000 U/ml) restriction enzyme were added to an eppendorf tube, mixed gently and incubated at 37°C for 1-2 h. Digests were visualised on 1% agarose gels containing 0.01 µg/µl ethidium bromide at 110V for 1 h.

4.2.15 Wizard® *Plus* SV MiniPrep DNA Purification System

When the insert was confirmed present with the miniprep, original cultures were regrown in LB broth overnight at 37°C for 16-18 h. Samples were purified for DNA quantitation and real-time PCR using the Wizard® *Plus* SV MiniPrep kit following the manufacturer's instructions for the centrifugation protocol. Briefly 1-5 ml of the confirmed bacterial culture, containing the inserts within the vector was harvested by centrifugation at 10,000 g/rcf (relative centrifugal force) for 5 min. The supernatant was aspirated and excess media removed. 250 µl of cell resuspension solution was added and samples were vortexed to thoroughly resuspend the pellet. 250 µl of cell lysis solution was added, tubes were inverted 4 times and incubated at RT for 5 min to allow clearing of the cell suspension. 10 µl of alkaline protease solution (Wizard® *Plus* SV MiniPrep kit) was added, samples were inverted 4 times and incubated at RT for 5 min. 350 µl of Wizard® *Plus* SV neutralisation solution was added and the tubes were

inverted 4 times. The bacterial lysates were centrifuged at 14,000 g for 10 min at RT. The cleared lysates were transferred to spin columns in 2 ml collection tubes and centrifuged for 1 min at RT. The spin columns were removed and the eluate was discarded. The spin columns were reinserted into the collection tubes and 750 µl of column wash solution (diluted with 95% ethanol) was added. Samples were centrifuged at RT for 1 min and the eluate was discarded. This step was repeated with 250 µl of column wash solution. Samples were centrifuged at 14,000 g for 2 min at RT. The spin columns were transferred to new 1.5 ml centrifuge tubes and the plasmid was eluted in 100 µl of Nuclease-free water. Samples were centrifuged at 14,000 g for 1 min and purified DNA was stored at -20°C.

4.2.16 Wizard® *Plus* MidiPrep DNA Purification System

The MidiPrep purification system was used to purify a larger volume of plasmid DNA. 100 ml of cultured cells was centrifuged at 10,000 g for 10 min at 4°C. The supernatant was discarded. The cell pellet was completely resuspended in 3 ml of cell resuspension solution, 3 ml of cell lysis solution was added and the tube was inverted 4 times. Once the suspension was clear, 3 ml of neutralisation solution was added and the tube was mixed by inversion 4 times. The sample was centrifuged at 14,000 g for 15 min at 4°C. The supernatant was decanted into a fresh tube. 10 ml of resuspended Wizard® Midipreps DNA purification resin was added and the solution was mixed. The midicolumn tip was inserted into the vacuum manifold port. The resin/DNA mixture was transferred into the midicolumn and a vacuum of 15 inches of HG was applied to pull the resin/DNA mixture into the midicolumn. When the sample passed through the column, the vacuum was broken at source. An ENDA$^-$ strain (TOP 10) was used, eliminating the need for the addition of 40% isopropanol/4.2M guanidine hydrochloride

solution. 15 ml of column wash solution was added to the column and a vacuum was applied to pull the solution through. The vacuum was broken at source and another 15 ml of wash solution was added to the column with a vacuum. A vacuum was applied for a further 30 sec after the solution was pulled through the column so as to dry the resin. The midicolumn was detached from the vacuum and the reservoir was separated from the column using a scissors. The column was transferred to a fresh 1.5 ml eppendorf tube and centrifuged at 10,000 g for 2 min. The column was placed in a new tube and 300 µl of preheated (65-70°C) nuclease-free water was added to the column which was allowed to sit for 1 min. The column was centrifuged at 10,000 g for 20 sec and the column was discarded. The sample was centrifuged at 10,000 g for 5 min and the DNA-containing supernatant was transferred to a fresh tube and stored at -20°C.

4.2.17 Cloned DNA Quantitation

The DNA concentration of cloned samples was quantified using the BIO-RAD VersaFluor™ Flourometer and Fluorescent DNA Quantitation Kit using Hoechst 33258 (BIO-RAD, California, USA). The 360 nm excitation filter and 460 nm emission filter were inserted, the gain was set to MED and the range was set to read 1000. DNA concentration was determined in the range of 20-1000 ng using a Hoechst 33258 concentration of 0.1 µg/ml. The standard curve was set up by labelling seven 12.5 mm (OD (optical density)) cuvettes. 2 ml of Hoechst dye was added to each cuvette and calf thymus DNA was added as described in Table 4.2. Cuvette 7 was blank and contained no DNA. The fluorometer was set to zero with the blank. Cuvette 2 was placed into the instrument and after 5-10 sec the RFU (Relative Fluorescence Units) reading was recorded. All standards were read in the same manner and an average was calculated (Table 4.3). Samples were read after all standards in duplicate.

Table 4.2 20 ng - 100 ng DNA Range for Hoechst 33258 Quantification

Cuvette	Total DNA (ng)	DNA Stock Solution	DNA Volume	0.1 ug/ml Hoechst Dye
1	1000	100 µg/ml	10 µl	2 ml
2	500	100 µg/ml	5 µl	2 ml
3	200	100 µg/ml	2 µl	2 ml
4	100	10 µg/ml	10 µl	2 ml
5	50	10 µg/ml	5 µl	2 ml
6	20	10 µg/ml	2 µl	2 ml
7	Blank	____	____	2 ml

Table 4.3

Average Absorbance Values for *Vibrio harveyi* and *Perkinsus olseni* Standards

Total DNA Concentration (ng)	Mean *Vibrio harveyi* RFU	Mean *Perkinsus olseni* RFU
20	15	17.5
50	27	57.5
100	67.5	95
200	185	210
500	516	655
1000	997	1200

Two standard curves were generated by plotting the total DNA concentration (ng) versus the RFU values for *Vibrio harveyi* and *Perkinsus olseni* (Fig. 4.5, Fig. 4.6).

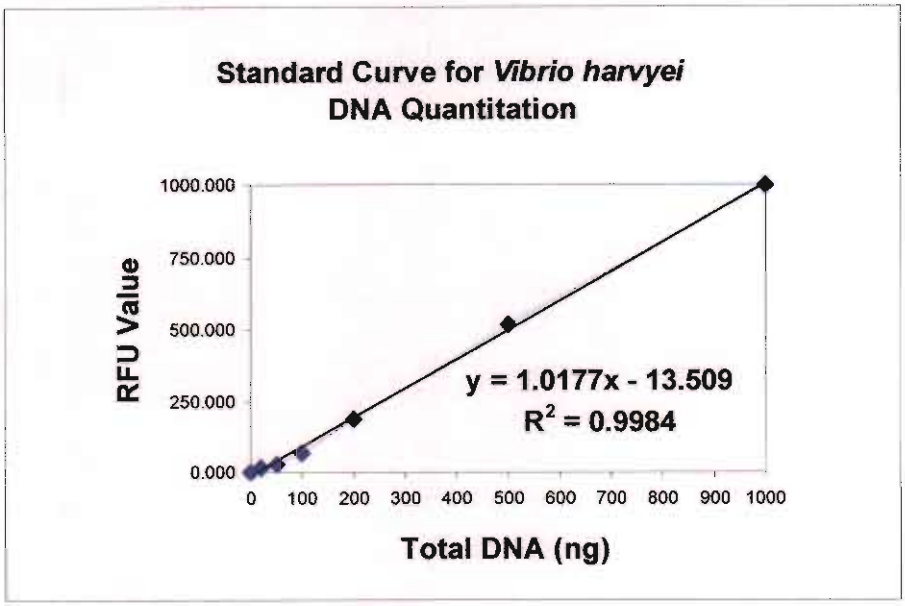

Figure 4.5. Plot of DNA standard curve for *Vibrio harveyi* quantitation with total DNA concentration (ng) versus RFU values.

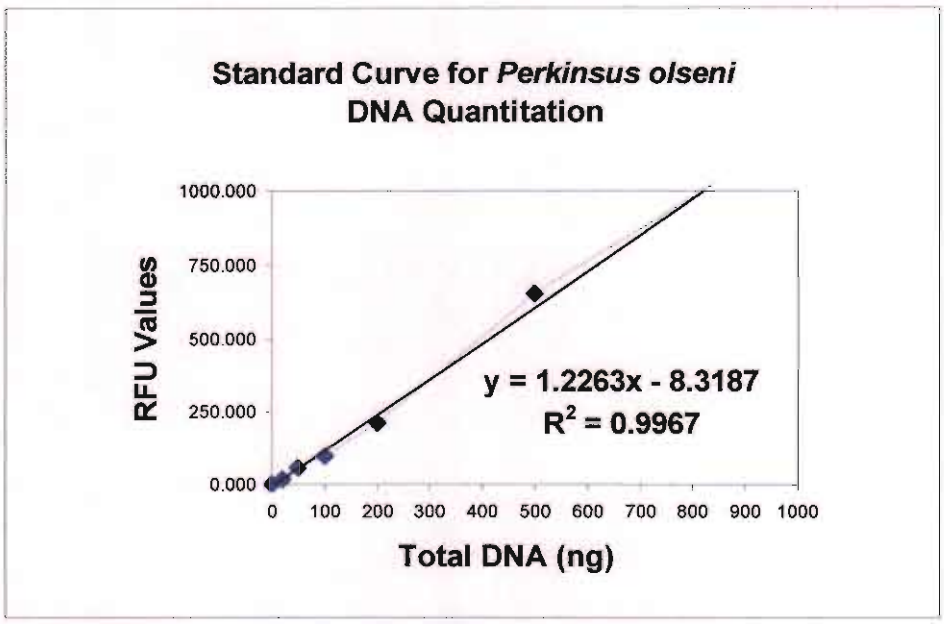

Figure 4.6. Plot of DNA standard curve for *Perkinsus olseni* quantitation with total DNA concentration (ng) versus RFU values.

From these curves the unknown protein concentrations of the *Vibrio harveyi* and *Perkinsus olseni* samples were determined using the formula Y = MX + C, where Y is the absorbance, M is the slope of the line, X is the protein concentration and C is a

constant by linear regression. The concentration of the *Vibrio harveyi* cloned DNA sample was 339.88 ng/µl (0.34 µg/µl) and the concentration of the *Perkinsus olseni* cloned DNA sample was 243.34 ng/µl (0.24 µg/µl). Using these concentrations, the copy number (number of cells per µl) for each sample was calculated per µg of total DNA as follows:

Copy number was calculated using the following formula:

$$M = \left(n \right) \left(\frac{1 \text{ mole}}{6.023 \times 10^{23}} \right) \left(\frac{660 \text{ g}}{\text{mole}} \right)$$

M = Mass of one plasmid molecule

n = Total size of plasmid and + insert

Avogadro's number = 6.023e23 molecules/1 mole

Average molecular weight of a double stranded DNA molecule = 660 g/mole.

Calculation of copy number for *Vibrio harveyi* cloned samples:

$$M = \left(\begin{array}{c} 3900bp + 413\ bp \\ \text{plasmid + insert} \end{array} \right) \left(1.096e^{-21} \right) = 4.72e^{-18}\ g = 1\ copy.$$

$$\text{Total DNA conc.} = \left(\frac{339.88\ ng/\mu l}{5\ \mu l\ \text{(vol. added to cuvette)}} \right) = 67.97\ ng/\mu l.$$

$0.06797e^{-6}$ g/µl (Total DNA) ÷ $4.72e^{-18}$ g (1 copy) = $1.438e^{10}$ copies per µl or $1.4e^{10}$ copies per µl.

Calculation of copy number for *Perkinsus olseni* cloned samples:

$$M = \left(\begin{array}{c} 3900bp + 265\ bp \\ \text{plasmid + insert} \end{array} \right) \left(1.096e^{-21} \right) = 4.57e^{-18}\ g = 1\ copy.$$

$$\text{Total DNA conc.} = \left(\frac{243.34\ ng/\mu l}{5\ \mu l\ \text{(vol. added to cuvette)}} \right) = 48.67\ ng/\mu l.$$

$0.04867e^{-6}$ g/µl (Total DNA) ÷ $4.57e^{-18}$ g (1 copy) = $1.106e^{10}$ copies per µl or $1.1e^{10}$ copies per µl.

4.2.18 Real-time PCR for Detection and Quantitation of *Vibrio harveyi*

Real-time PCR for the detection and quantitation of *Vibrio harveyi* was optimised using the VH-1/2 primers, as used for standard PCR (Fig. 4.1) and the *LightCycler*® DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany). Magnesium chloride titrations for optimal amplification were determined in this study and a concentration of 2 mM was selected for use. An aliquot of 1 µl of DNA was used in a 10 µl *Lightcycler*® DNA Master SYBR Green I reaction with a final concentration of 2 mM $MgCl_2$, 100 ng/ul of each primer and 1X SYBR Green I enzyme mix. The reaction was run on a *Lightcycler*® Instrument (Roche Diagnostics) under the following conditions: An initial 2 min at 95°C, followed by 40 cycles of 2 sec of denaturation at 95°C, 10 sec of annealing at 65°C and 5 sec of extension at 72°C. A melt curve was acquired by heating the product at 20°C/s to 95°C, cooling it at 20°C/s to 65°C, and slowly heating it at 0.2°C/s to 95°C. The temperature was cooled to 40°C and held for 5 sec.

4.2.19 Real-time PCR for Detection and Quantitation of *Perkinsus olseni*

Real-time PCR for detection and quantitation of *Perkinsus olseni* was optimised using the ITSR1 primers, as used for standard PCR (Fig. 4.3) and the *LightCycler*® DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany). Magnesium chloride titrations for optimal amplification were determined in this study and a concentration of 3 mM was selected for use. An aliquot of 1 µl of DNA was used in a 10 µl *Lightcycler*® DNA Master SYBR Green I reaction with a final concentration of 3 mM $MgCl_2$, 100 ng/ul of each primer and 1X SYBR Green I enzyme mix. The reaction was run on a *Lightcycler*® Instrument (Roche Diagnostics) under the following conditions: An initial 2 min at 95°C, followed by 40 cycles of 2 sec of denaturation at

95°C, 10 sec of annealing at 65°C and 5 sec of extension at 72°C. An extra step of 85°C for 10 sec was added after the annealing step to reduce the effect of primer dimers. A melt curve was acquired by heating the product at 20°C/s to 95°C, cooling it at 20°C/s to 65°C, and slowly heating it at 0.1°C/s to 95°C. The temperature was cooled to 40°C and held for 5 sec.

4.2.20 Analysis of Results

Quantification of amplified product was done on a cycle-by-cycle basis through the acquisition of a fluorescent signal. Amplification of the product was visualized in the quantification curve analysis which measures the increase in fluorescence through binding of the DNA to the fluorophore SYBR Green I dye. The specificity of the amplified products was confirmed by a melt curve analysis in which positive samples showed a specific peak with a melt temperature of approximately 89°C with the VH-1/2 primers and 86°C with the ITSR1/2 primers. Specificity was further confirmed by analysing the PCR products on 2% agarose gels as previously described. Standard curves were created using the *Lightcycler*® (Roche Molecular Biosystems) equipment so that crossing points of unknown samples could be compared with the standard curve, which allows for the amount of target DNA in the unknown sample to be determined.

4.3 RESULTS

4.3.1 Detection of *Vibrio harveyi* by Standard PCR

The specificity of the VH-1/2 PCR was tested by isolating DNA from isolates of *Vibrio harveyi*, *Vibrio anguillarum*, *Vibrio alginolyticus*, *Vibrio splendidus*, *Vibrio tapetis* and *Vibrio tubiashii*. Amplicons of 413 bp were observed from DNA extracted from all *Vibrio harveyi* isolates tested using the VH-1/2 primers (Fig. 4.7). A 413 bp amplicon was also detected from the *Vibrio alginolyticus* isolate but not in the other *Vibrio* species tested (Fig. 4.7).

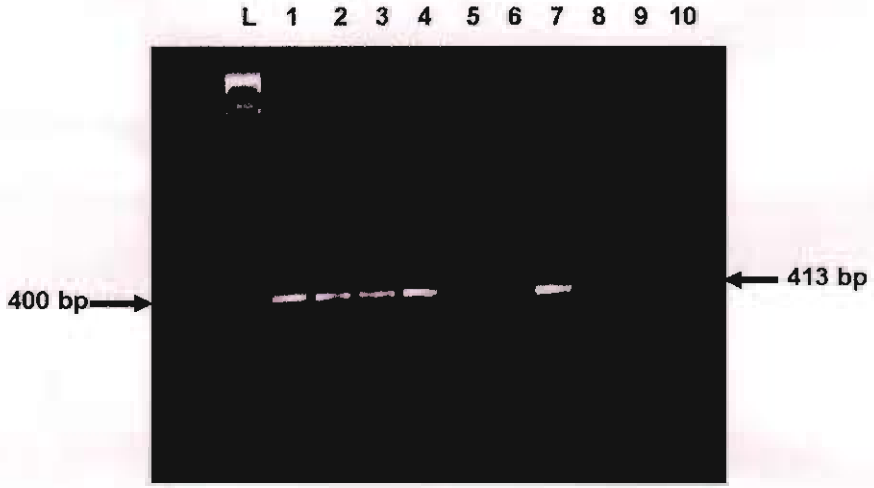

Figure 4.7. PCR amplification of the *Vibrio harveyi* 16S rRNA gene of *Vibrio* isolates generated using the VH-1/2 primers. L = 100 bp ladder; Lanes 1-4 = *V. harveyi*; Lane 5 = Negative control; Lane 6 = *V. anguillarum*; Lane 7 = *V. alginolyticus*; Lane 8 = *V. splendidus*; Lane 9 = *V. tapetis*; Lane 10 = *V. tubiashii*.

As often only preserved tissue is available the PCR was tested on DNA isolated from alcohol-fixed abalone tissues infected with *Vibrio harveyi*. A 413 bp amplicon was detected in DNA isolated from three alcohol-fixed tissue samples of abalone infected with *V. harveyi* (Fig. 4.8).

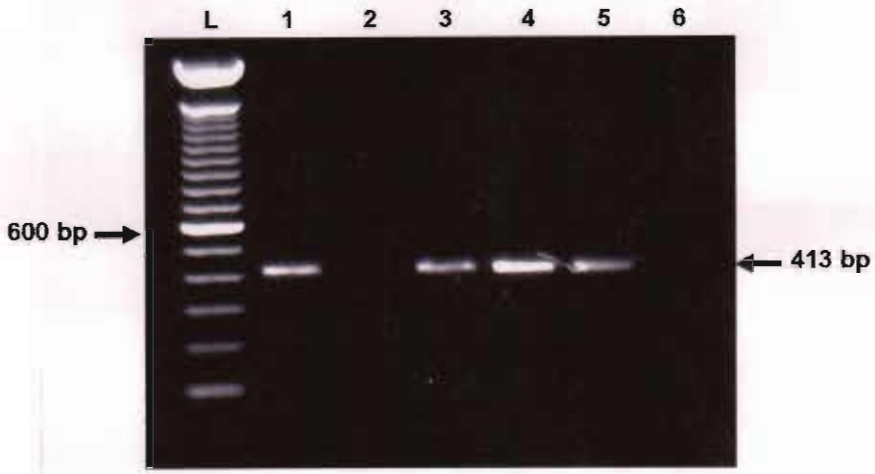

Figure 4.8. PCR amplification of *Vibrio harveyi* from *Haliotis tuberculata* tissues using the VH-1/2 primers. L = 100 bp ladder; Lane 1= *V. harveyi* positive control; Lane 2 = Uninfected *Haliotis tuberculata*; Lanes 3-5 = *Vibrio harveyi* infected *Haliotis tuberculata*; Lane 6= Negative control.

4.3.2 Multiplex PCR for *Vibrio harveyi* Detection and Abalone Actin Amplification

To control for DNA integrity in alcohol fixed tissues the amplification of an abalone actin gene was included in the PCR. The multiplex PCR simultaneously amplified a 413 bp *Vibrio* product and a 155 bp abalone actin gene product from 3 alcohol-fixed abalone tissue samples infected with *Vibrio harveyi* (Fig. 4.9).

This multiplex was also applied to *Vibrio harveyi* infected paraffin embedded abalone tissues. Successful amplification of both the 413 bp and 155 bp bands was observed in one sample (Fig. 4.10). In lanes 3 and 5 the 413 bp band did not amplify as a result of low DNA yield, however the lower molecular weight band of 155 bp did amplify in these two samples.

Figure 4.9. Multiplex PCR: *Haliotis tuberculata* alcohol-fixed tissues infected with *Vibrio harveyi* amplified for the 16S rRNA gene (VH-1/2) and abalone actin DNA (ACTIN-1/2). L = 100 bp ladder; Lane 1 = *Vibrio harveyi* positive control; Lane 2 = Uninfected *Haliotis tuberculata*; Lanes 3-5 = *Vibrio harveyi* infected *Haliotis tuberculata* tissues; Lane 6 = Negative control.

Figure 4.10. Multiplex PCR: *Haliotis tuberculata* paraffin embedded tissues infected with *Vibrio harvyei* amplified for the 16S rRNA gene (VH-1/2) and abalone actin DNA (ACTIN-1/2). L = 100 bp ladder; Lane 1 = *Vibrio harveyi* positive control; Lane 2 = Uninfected *Haliotis tuberculata*; Lanes 3-5 = *Vibrio harveyi* infected *Haliotis tuberculata* paraffin embedded tissues; Lane 6 = Negative control.

4.3.3 Quantitative Real-time PCR for *Vibrio harveyi*

A real-time quantitative method for the detection of *Vibrio harveyi* in abalone tissues was developed. *Vibrio harveyi* PCR products were cloned using the pCR® 2.1 TOPO vector. The cloned product was digested using EcoR1 enzyme to verify the presence of the insert within the plasmid (Fig. 4.11). Cloned *V. harveyi* DNA was quantified using the VersaFluor™ Fluorometer and the copy number was calculated as $1.4e^{10}$.

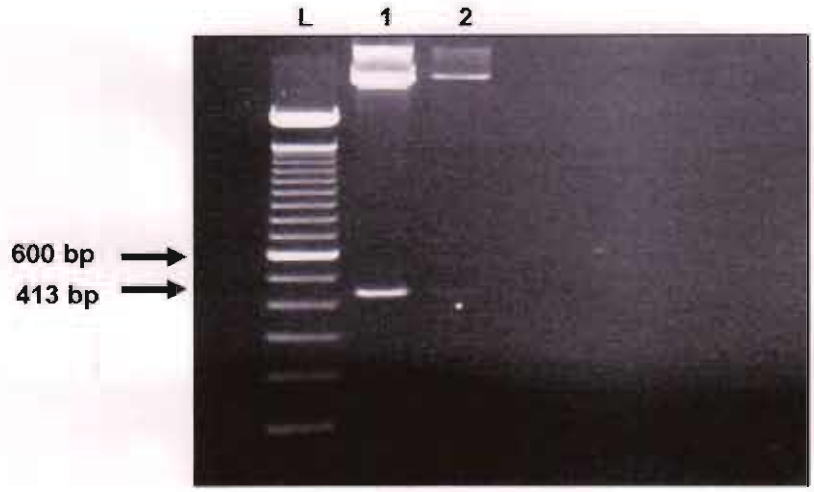

Figure 4.11. Restriction digest of cloned *Vibrio harveyi* PCR product. L = 100 bp ladder; Lane 1 = Plasmid at 3.9 kb and *V. harveyi* insert at 413 bp; Lane 2 = Negative control - plasmid with no insert.

Target *V. harveyi* DNA was detected in the *Lightcycler*® real-time PCR method with DNA copy numbers of 10^7 down to 10^3 (Fig. 4.12). A standard curve was created from cloned standards of *V. harveyi* (Fig. 4.13) enabling the quantification of bacterial load in infected samples.

Of the three alcohol-fixed *H. tuberculata* tissue samples infected with *V. harveyi*, two samples (T1; T2) were amplified by real-time PCR with *Vibrio* copy numbers of 10^4 (Fig. 4.12). The third sample (T3) failed to amplify probably owing to low pathogen numbers. Three *H. tuberculata* paraffin embedded samples infected with *V. harveyi*

were also analysed using SYBR Green real-time PCR. One out of three samples amplified successfully with a *Vibrio* copy number of 10^3.

Melt curve analysis was performed to confirm PCR product identity and differentiate specific product from primer-dimers (Fig. 4.14). The T_m observed with the *V. harveyi* cloned samples and the *V. harveyi* infected abalone samples was approximately 89°C. Primer-dimers were observed at the lower temperature of 76°C.

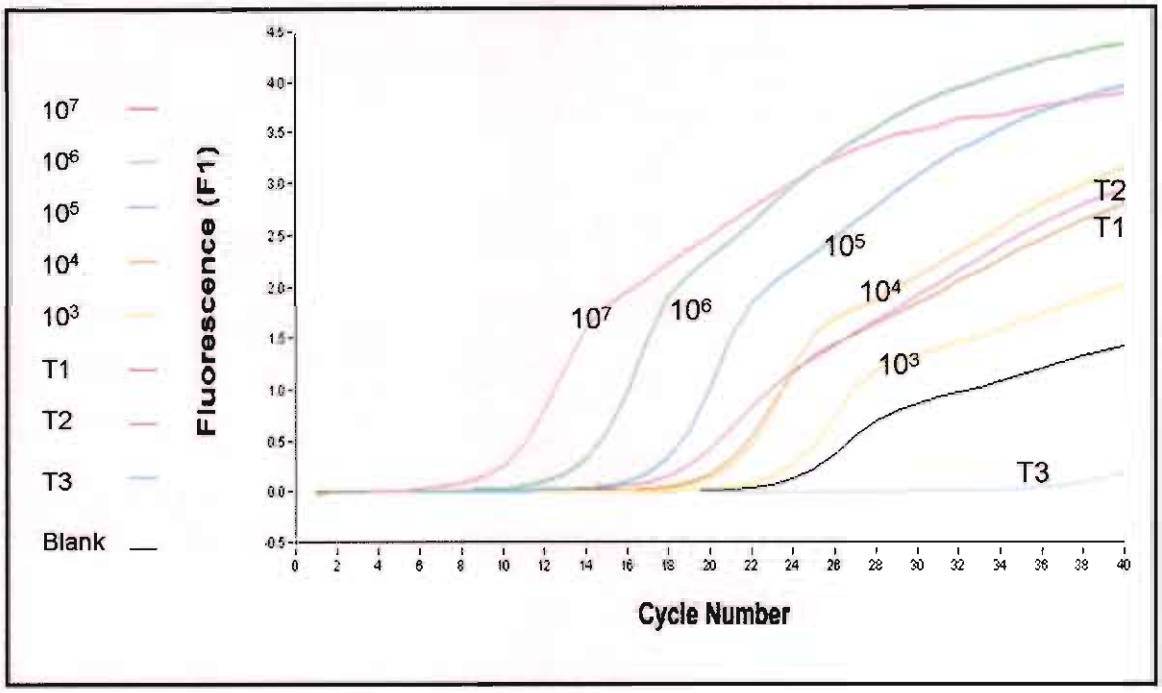

Figure 4.12. *LightCycler*® real-time PCR amplification plot showing increases in fluorescence from cloned *V. harveyi* standards (10^7-10^3) and 3 *H. tuberculata* alcohol fixed tissue samples (T1; T2; T3) infected with *V. harveyi*.

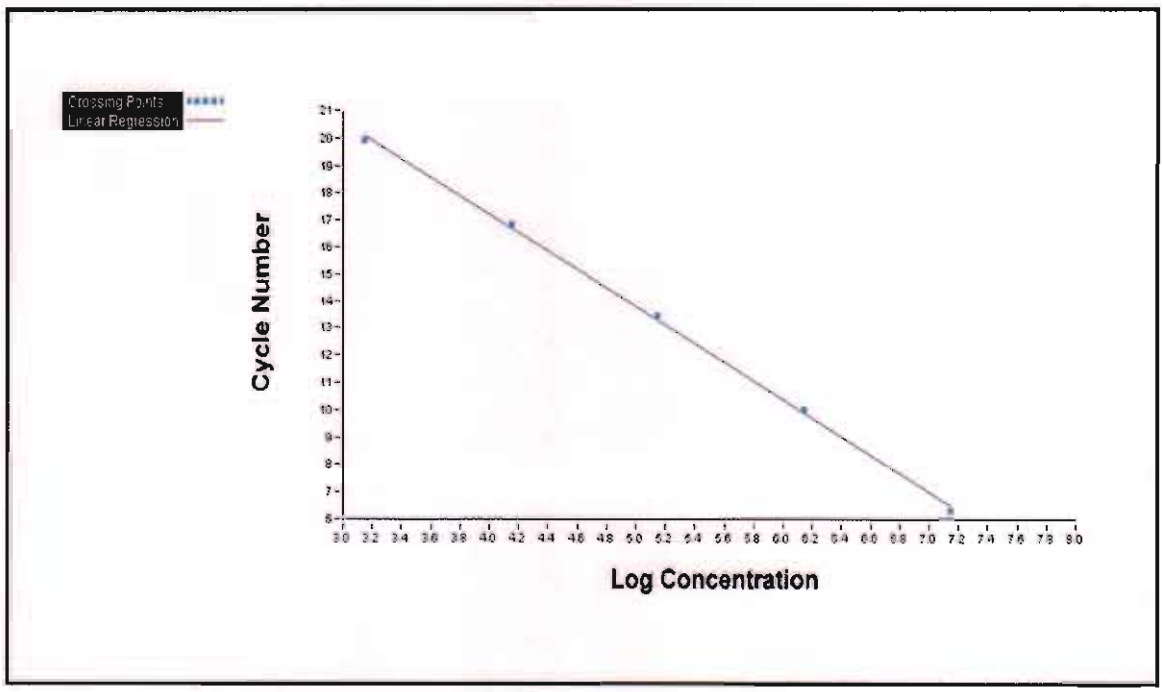

Figure 4.13. *LightCycler*® real-time PCR standard curve of cloned *V. harveyi* standards for the quantitation of *V. harveyi* copy numbers.

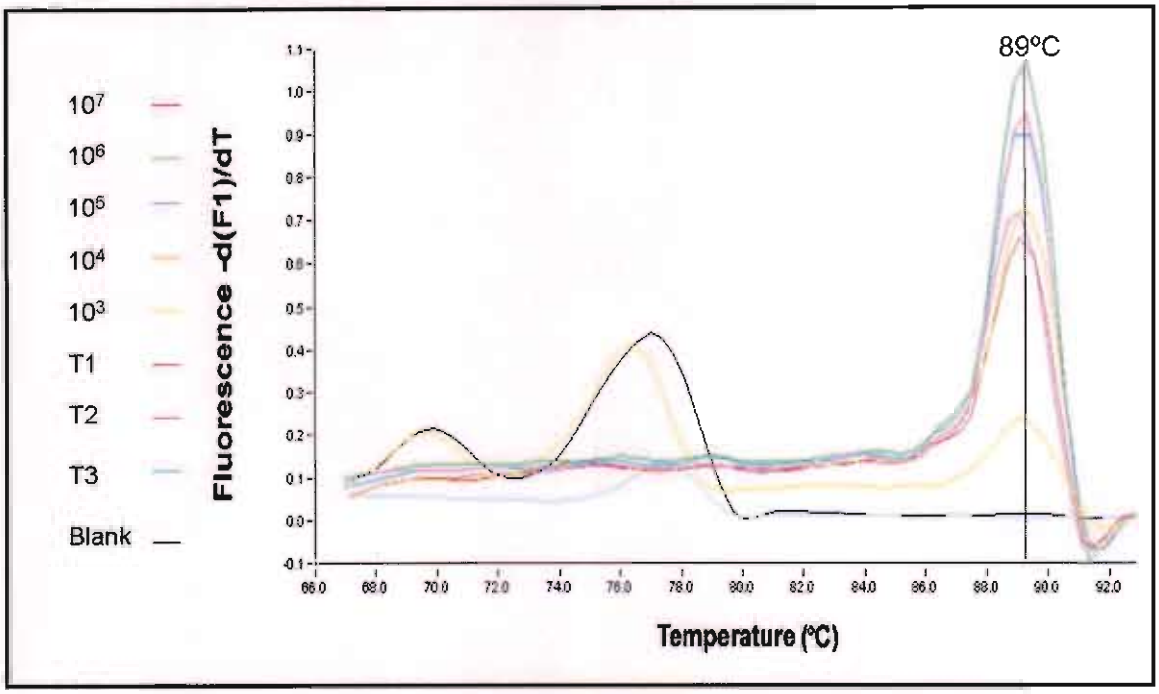

Figure 4.14. *LightCycler®* real-time PCR melt curve analysis of cloned *V. harveyi* standards (10^7-10^3) with *H. tuberculata* alcohol fixed tissue samples (T1; T2; T3) infected with *V. harvyei* with a T_m of 89°C.

4.3.4 Detection of *Perkinsus olseni* by Standard PCR

In this study new primers were designed which amplify part of the ITS region of the *Perkinsus olseni* genome sequence. These primers were incorporated into a novel PCR which detected *P. olseni* in pure isolates and *P. olseni* in paraffin embedded shellfish tissues. Successful amplification of *P. olseni* DNA from pure isolates was observed in a standard PCR with the ITS-1/2 primers generating a 265 bp band (Fig. 4.15). A 265 bp band was also generated from paraffin embedded clam (*Ruditapes decussatus*) tissues infected with *P. olseni*. Intense bands were observed in two samples (Lanes 3-4) owing to higher pathogen load as confirmed by histological screening.

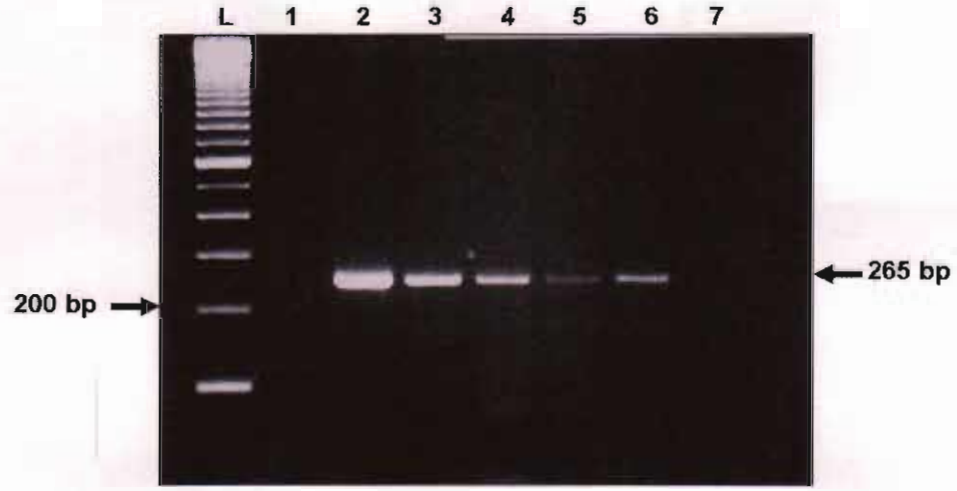

Figure 4.15. PCR amplification of *Perkinsus olseni* using the ITS-1/2 primers. L = 100 bp ladder; Lane 1 = Uninfected *Haliotis discus hannai*; Lane 2 = *P. olseni* isolate; Lanes 3-6 = *P. olseni* infected *Ruditapes decussatus* tissues; Lane 7 = Negative control.

4.3.5 Quantitative Real-time PCR for *Perkinsus olseni*

Real-time PCR was developed to detect and quantify *Perkinsus olseni*. *P. olseni* PCR products were cloned using the pCR® 2.1 TOPO vector. A restriction digest, using EcoR1 enzyme, was carried out to verify the presence of the insert within the plasmid (Fig. 4.16). Cloned *P. olseni* DNA was quantified using the VersaFluor™ Fluorometer and copy number was calculated as $1.1e^{10}$.

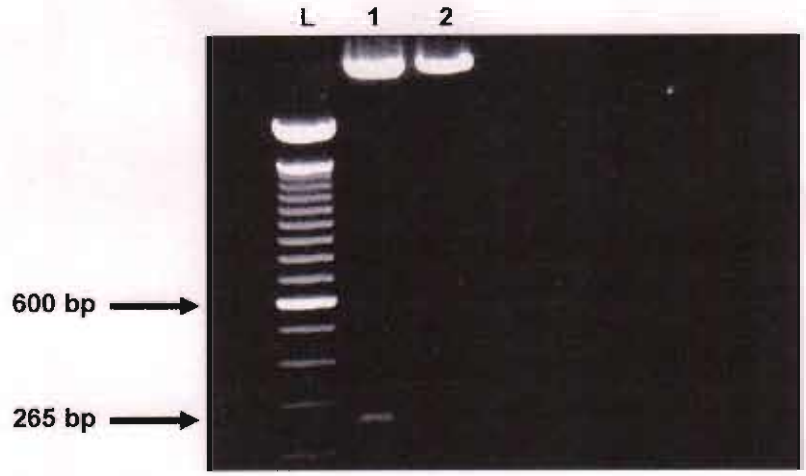

Figure 4.16. Restriction digest of cloned *Perkinsus olseni* PCR product. L = 100 bp ladder; Lane 1 = Plasmid at 3.9 kb and *P. olseni* insert at 265 bp; Lane 2 = Negative control - plasmid with no insert.

Target *Perkinsus olseni* DNA was detected in the real-time PCR method with DNA copy numbers of 10^8 to 10^2 (Fig. 4.17). A standard curve was created from cloned standards of *P. olseni* using *Lightcycler*® SYBR Green PCR (Roche Molecular Biosystems) (Fig. 4.18). Melt curve analysis was also performed during this PCR to verify the amplification of the correct PCR product (Fig. 4.19). A T_m of 87°C was observed with *P.olseni* standards which was further confirmed by gel electrophoresis. Primer-dimers formed at a lower temperature of 80-81°C.

Four paraffin embedded samples of *R. decussatus* infected with *P. olseni* which amplified with conventional PCR were analysed on the *Lightcycler*® SYBR Green PCR system alongside the *P. olseni* cloned standards. The *P. olseni* product failed to amplify with SYBR Green PCR. Various experiments using a *LightCycler*® FastStart SYBR Green kit (Roche Diagnostics), different quantities of DNA template, a range of dilutions of the DNA template and different cycle conditions were conducted in an attempt to optimise quantitative real-time SYBR Green PCR for the detection and

enumeration of *P. olseni* in paraffin embedded tissues. It was concluded that more work using alcohol-fixed samples infected with *P. olseni* would be required.

Serial dilutions (1:10-1:10000) of DNA extracted from the *P. olseni* isolate were set up and tested with *Lightcycler*® SYBR Green PCR alongside cloned *Perkinsus* standards. The system successfully amplified target *Perkinsus* DNA (Fig. 4.20) and a melt curve with a T_m of 87°C was observed with both the cloned standards and the serial dilutions of the *P. olseni* test samples (Fig. 4.21).

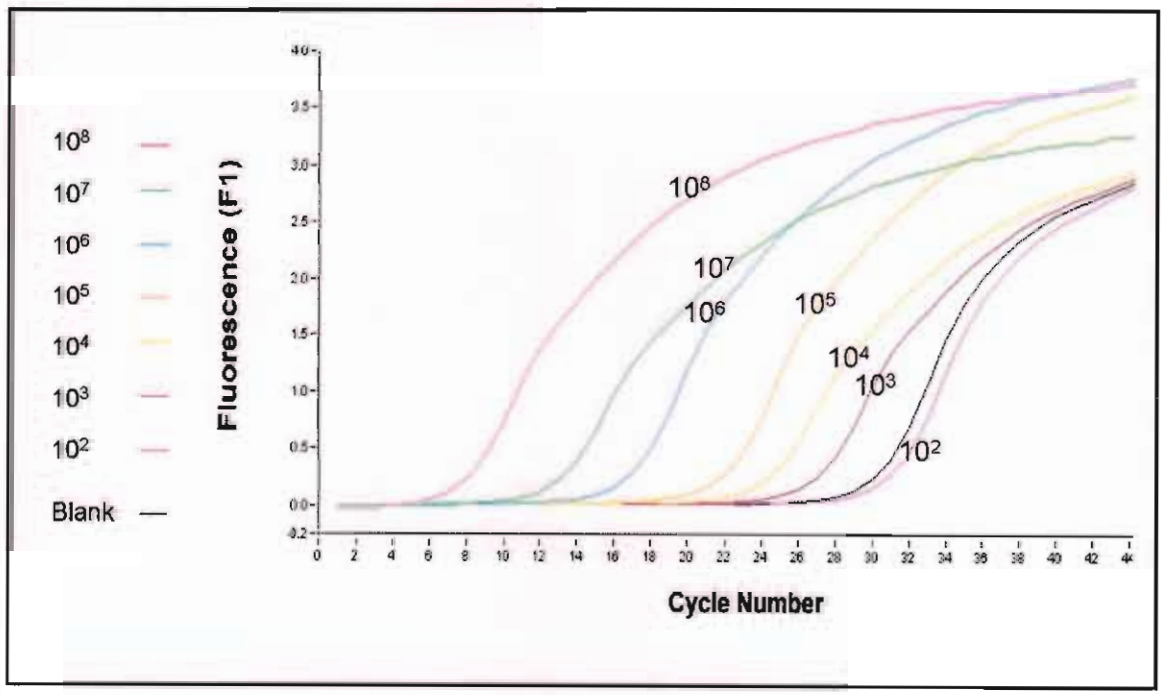

Figure 4.17. *LightCycler*® real-time PCR amplification plot showing increases in fluorescence from cloned *P. olseni* standards (10^8-10^2).

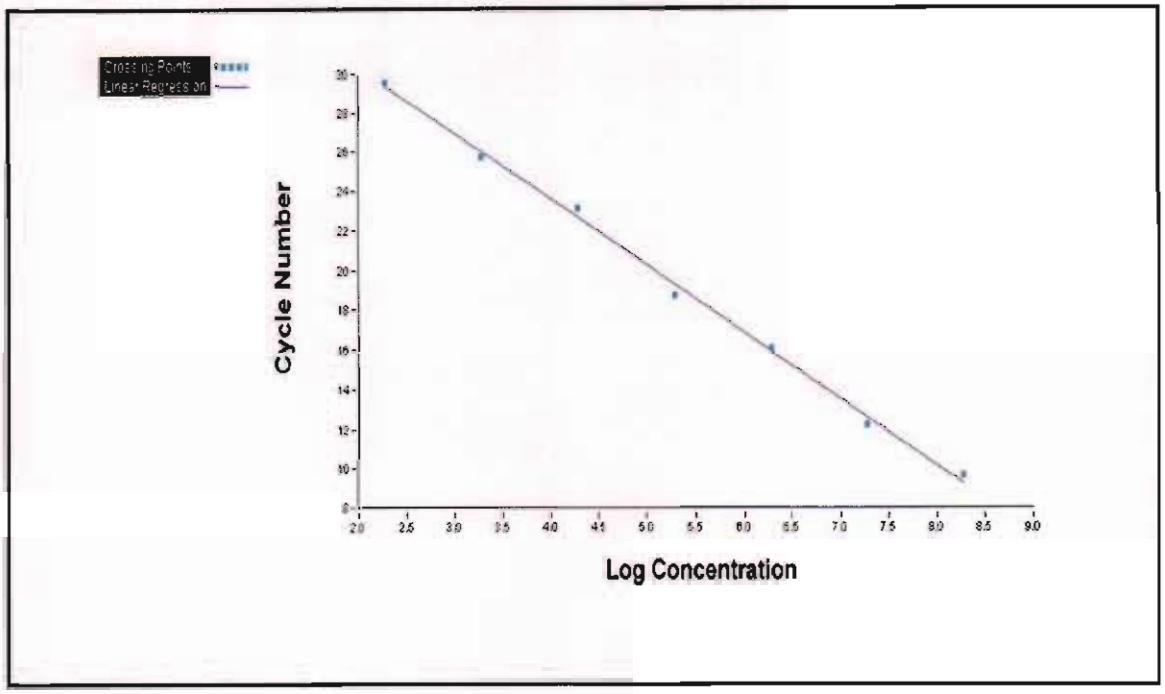

Figure 4.18. *LightCycler*® real-time PCR standard curve of cloned *P. olseni* standards for the quantitation of *P. olseni* copy numbers.

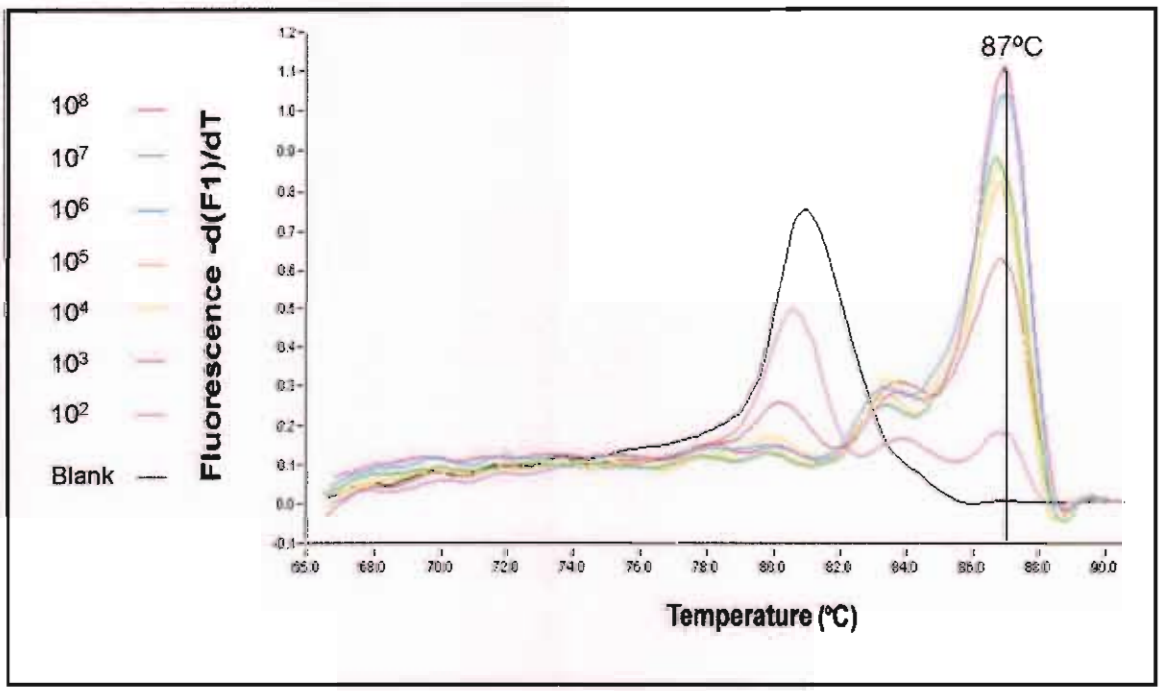

Figure 4.19. *LightCycler*® real-time PCR melt curve analysis of cloned *P. olseni* standards (10^8-10^2) with a T_m of 87°C.

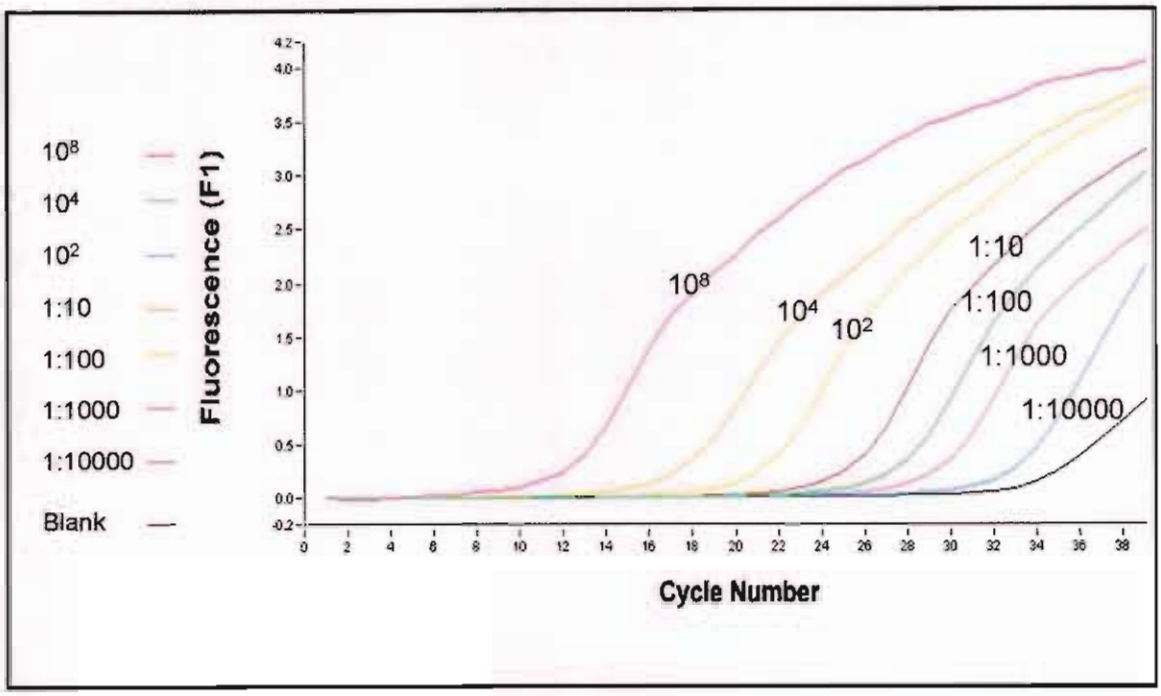

Figure 4.20. *LightCycler*® real-time PCR amplification plot showing increases in fluorescence from cloned *P. olseni* standards (10^8-10^2) and serial dilutions (1:100-1:10000) of DNA extracted from a *P. olseni* isolate.

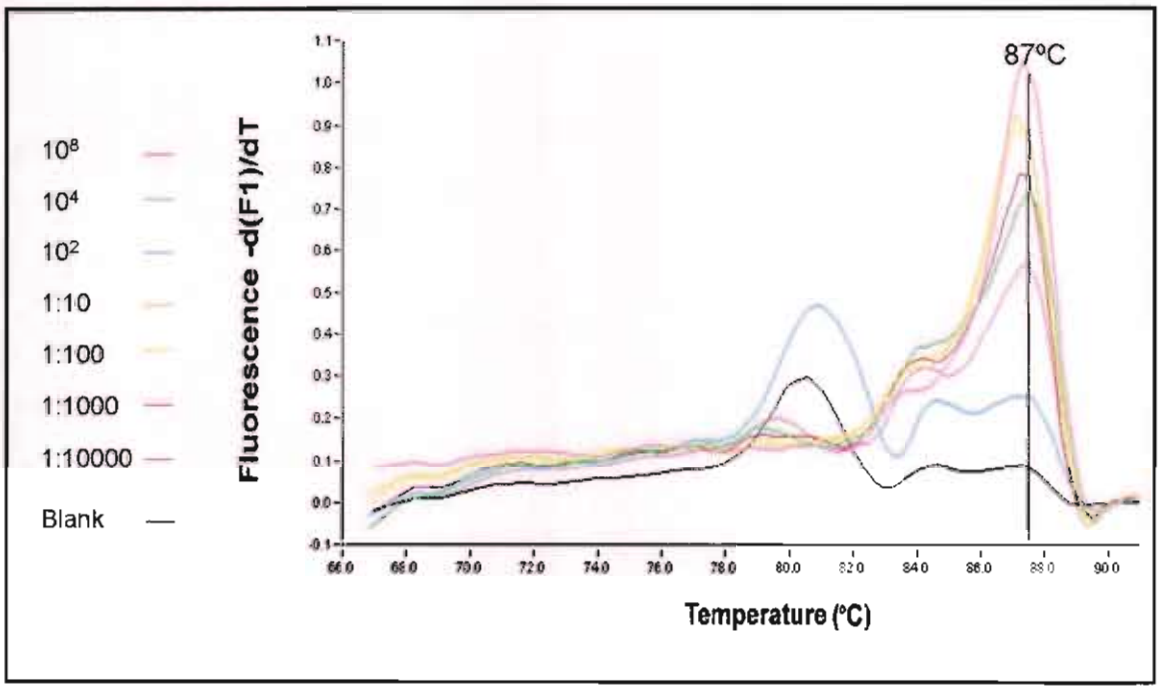

Figure 4.21. *LightCycler*® real-time PCR melt curve analysis of cloned *P. olseni* standards (10^8-10^2) and serial dilutions (1:100-1:10000) of DNA extracted from a *P. olseni* isolate with a T_m of 87°C.

4.4 DISCUSSION

The rising popularity of abalone aquaculture has resulted in increased awareness of infectious diseases. There are many risks associated with the uncontrolled transfer and introduction of live aquatic organisms to culture facilities. With the establishment of new aquaculture industries the transplantation of uninfected larval abalone is an essential requirement (Bower et al. 1994; Bower 2003). Abalone shellfish are susceptible to many pathogenic diseases such as Vibriosis, Perkinsosis, Amyotrophia, and Haplosporidiosis (Bower 1994). As abalone aquaculture is a relatively new phenomenon in Ireland there have been no disease outbreaks to date. Production of abalone is currently small scale but is successfully growing in size and there are more disease risks associated with large scale production. New pathogens are constantly emerging so the translocation of molluscs, whether domestic or international, always carries the risk of pathogen introduction especially when facilities are disease free. It is imperative that strict guidelines on molluscan translocation, as set out in the ICES (International Council for the Exploration of the Sea) protocol for the introduction of species (ICES 1998), are maintained and protocols such as rapid diagnostic techniques are developed to curtail the spread of disease.

Vibrio harveyi and *Perkinsus olseni* are two major pathogens of abalone causing serious disease in both wild and cultured stocks. With the development of nucleic acid-based methods for pathogen detection it has become evident that the 16S region of the bacterial rRNA gene and the ITS region of the eukaryotic rRNA gene are the most commonly employed targets for the discrimination and detection of these two species. The comparison of rRNA sequences is a powerful tool for deducing the phylogenetic and evolutionary relationships among bacteria, archaebacteria and eukaryotic organisms

(Weisburg et al. 1991). Other gene sequences exist such as actins, tublins, superoxide dismutases and serine proteases (Villalba et al. 2004) and information on these sequences will become available when they have been thoroughly determined. Phylogenetic studies, species comparisons and analysis of intraspecific variation will benefit from additional information generated about these sequences.

4.4.1 PCR Detection of *Vibrio harveyi*

Vibrio carchariae, which is a junior synonym of *Vibrio harveyi*, was amplified with the VH primers in this study as expected. Pedersen et al. (1998) revealed similar ribotyping patterns (which reveal genetic variations) of *Vibrio harveyi* and *V. carchariae* and observed 88% DNA binding between these two species rendering them the same species.

Vibrio harveyi is a serious pathogen of *Haliotis tuberculata*, which has been confirmed by Nicolas et al. (2002) through an experimental reproduction of the disease, whereby the pathogen was introduced into a water body containing a stock of abalone. Following exposure to the pathogen, 85% of the abalone population died.

In this study, a PCR method to amplify part of the *Vibrio harveyi* 16S rRNA gene sequence (adapted from Oakey et al. 2003) was used to detect DNA isolated from *Vibrio harveyi* bacterial cultures. This PCR method was optimised to detect *Vibrio harveyi* in DNA isolated from alcohol preserved tissues and paraffin embedded tissues of *Haliotis tuberculata*. The PCR was extended to a multiplex PCR, by including primers to amplify an abalone actin gene as an internal control for DNA integrity. PCR techniques for the species-level identification of abalone shellfish have been developed

and actin genes have been isolated from abalone using PCR (Lee and Vacquier 1995; Sweijd et al. 1998; Elliott et al. 2002; Bryant et al. 2006). In the study presented here, newly designed primers for the amplification of a partial sequence of an abalone actin gene were successfully established.

The VH primers also amplified DNA extracted from *Vibrio alginolyticus* in this study but not any of the other species tested. *V. alginolyticus* is an identified pathogen of fish and shellfish and has been associated with mortality amongst abalone shellfish (Balebona et al. 1998; Lee et al. 2001; Robert-Pillot et al. 2002; Goméz-León et al. 2005). Oakey et al. (2003) developed the VH primers and found that it amplified part of the 16S rRNA gene of *V. alginolyticus* as well as *V. harveyi*. When the 413 bp sequences from both *V. harveyi* and *V. alginolyticus* were compared in other studies there was very little difference between them. Gene comparisons were not carried out in the study presented here. The results of Pedersen et al. (1998) show 65% DNA binding between these two type strains indicating why these two *Vibrio* species amplify with the VH primers. If necessary these two species can be phenotypically differentiated using conventional biochemical tests such as colonial morphology and the Voges-Proskauer (VP) test (Oakey et al. 2003).

There are many advantages to amplifying DNA from tissue and especially paraffin embedded samples as it is an invaluable resource for retrospective molecular studies (Wu et al. 2002). As observed here, pure strains of the bacterium produced excellent results with PCR amplification. The multiplex PCR was successful using fixed tissues, however with paraffin embedded tissues DNA yield was a limiting factor, as only one out of three samples produced the two amplicons. Poor integrity is usually associated

with DNA isolated from paraffin embedded tissues as a result of fixation and processing (Neubauer et al. 1992; Harris and Jones 1997). Multiplex PCR may be a more suitable method for pathogen detection from paraffin embedded samples, as the incorporation of a housekeeping gene will give a good indication of the integrity of the extracted DNA.

Nucleic acid-based techniques should play an integral part in the diagnosis of fish and shellfish diseases but despite the recent increase in the development of molecular techniques for rapid diagnosis of *Vibrio* spp. no validated method is available as yet. The results of this study may aid in developing molecular diagnostics in the marine arena to facilitate easy diagnosis and aid in disease surveillance.

In this study the sequences of the 16S rRNA gene of *Vibrio* spp. and the actin gene of *Haliotis* spp. were detected by multiplex PCR. This rapid and sensitive technique for the detection of this pathogen can be applied to whole tissues and paraffin-embedded tissues of diseased shellfish. This method has potential for application to the early diagnosis of *Vibrio* outbreaks.

4.4.2 PCR Detection of *Perkinsus olseni*

Perkinsus olseni has come to light as a serious pathogen of shellfish and in particular abalone shellfish. Outbreaks of *Perkinsus* spp. infection in commercially important molluscs including abalone have been recorded since 1981 (Lester and Davis 1981; O'Donoghue et al. 1991; Hamaguchi et al. 1998; Russell et al. 2004). In the period 1986-1992 around the Yorke Peninsula, South Australia, nine species of molluscs were found to be infected with *Perkinsus* spp. and four of those molluscs were *Haliotis* spp (Goggin and Lester 1995). Stress such as high temperature seems to be a factor

contributing to *Perkinsus* spp. outbreaks but laboratory trials revealed that even at lower temperatures of 15°C, *Perkinsus* caused mortality amongst abalone shellfish (Goggin and Lester 1995).

A new PCR using novel primers designed in this study targeting part of the ITS region of the rRNA gene of *P. olseni* was established. Nucleic acid-based techniques previously described for the detection of *Perkinsus* spp. (De la Herrán et al. 2000; Robledo et al. 2000; Elandalloussi et al. 2004) all amplify large DNA fragments which would not be suitable for PCR amplification of DNA from paraffin embedded shellfish samples. In this study primers were designed to amplify a 265 bp fragment from the ITS region of the *Perkinsus olseni* genome. This amplification method was applied to DNA extracted from pure isolates of *P. olseni* and four samples of *Ruditapes decussatus* infected with *Perkinsus olseni*. Successful amplification of the 265 bp fragment was achieved with the *P. olseni* isolates and the *P. olseni* infected paraffin-embedded clam samples.

As previously outlined, *Perkinsus olseni* and *Perkinsus atlanticus* are synonymous species with 98-99% similarity between their consensus sequences (Murrell et al. 2002). Other studies have also confirmed these species similarities. The ITS region of five *Perkinsus* spp. was investigated by Goggin (1994) which included *P. atlanticus* from Portuguese *Ruditapes decussatus* and *P. olseni* from Australian *Haliotis laevigata*. Both isolates had an identical sequence for the ITS1 region but the ITS2 differed by a small percentage. These minor discrepancies indicate that *P. olseni* and *P. atlanticus* are members of a single species which was confirmed by Robledo et al. (2000) who studied

the small subunit (SSU) and the non-transcribed spacer (NTS) of *P. atlanticus*. This synonymy is further confirmed by the results presented here.

The ability to amplify DNA extracted from paraffin embedded tissues has many advantages. In this study, all four *P. olseni* infected paraffin embedded clam samples amplified with the ITS primers. Of the four samples tested, two exhibited stronger bands with gel electrophoresis and prior histological staining revealed that these two host organisms were more heavily infected by the pathogen. PCR is very sensitive and accurate when using DNA extracted from pure isolates and alcohol-fixed tissues, and will be a rapid method useful as an adjunct to conventional histological investigations.

4.4.3 Quantitative Real-time PCR of *Vibrio harveyi* and *Perkinsus olseni*

Standard PCR is not inherently quantitative but real-time PCR for the detection of shellfish pathogens offers quantitative analysis while reducing the need for additional post-assay processing, resulting in rapid results (Harwood et al. 2004). Real-time PCR has proved to be highly specific in the detection of shellfish pathogens such as members of the *Vibrio* spp. (Harwood et al. 2004; Panicker et al. 2004). Real-time PCR using SYBR Green for the detection of *V. vulnificus* proved highly effective where eighty positive *V. vulnificus* strains were tested and found to be positive by the assay indicating its potential for incorporation into standard testing (Panicker et al. 2004). Audemard et al. (2004) have developed the first *Perkinsus* SYBR Green real-time PCR assay which detects and quantifies *Perkinsus marinus* in environmental water samples.

In this study two real-time SYBR Green PCR assays were developed for the detection and quantitation of *Vibrio harveyi* and *Perkinsus olseni* in alcohol-fixed and paraffin

embedded tissues. The real-time PCR assay for the detection of *V. harveyi* in abalone alcohol-fixed tissues successfully amplified the 413 bp region of the 16S rRNA gene and quantified pathogen load with a copy number of 10^4 cells. Real-time PCR of paraffin embedded samples was partially successful with one of three samples amplifying with this assay.

Real-time PCR was developed to detect and quantify *P. olseni* in infected shellfish samples. Due to the scarcity of *P. olseni* infected abalone samples, four *R. decussatus* paraffin embedded samples infected with *P. olseni* were tested with this real-time PCR method. Real-time PCR amplification of paraffin-embedded samples was not successful; however the method has been established and amplifies DNA from pure *Perkinsus* isolates and will be helpful for *Perkinsus* detection in alcohol-fixed or unfixed tissues.

Real-time PCR is well known for its sensitivity and rapidity in pathogen detection (Mackay 2004; Vadopalas et al. 2006). The real-time methods developed in this study can detect pathogens in the range of 10^{10}-10^2 cells per sample. Campbell and Wright (2003) described a TaqMan® real-PCR assay to detect DNA from pure cultures of *V. vulnificus* in oysters down to 10^2 CFU ml $^{-1}$ using a 6-carboxyfluorescein-labelled probe and a quencher dye (6-caroboxy-*N,N,N' N'*-tetramethylrhodamine). A detection level of 100 cells (10^2/µl DNA) of *V. vulnificus* from oyster tissues and Gulf of Mexico seawater was achieved in a real-time PCR assay using SYBR Green I (Panicker et al. 2004). Audemard et al. (2004) detected DNA concentrations of *P. marinus* as low as 3.3 × 10e-2 using SYBR Green real-time PCR. Two DNA extractions kits were compared and results showed that DNA recovery with the DNeasy Tissue Kit was greater than with

the QIAamp DNA Stool Mini Kit. To achieve real-time PCR that detects extremely low levels of pathogen load it may be necessary to carry out and compare several DNA extraction protocols.

While real-time PCR works well with alcohol-fixed tissues, paraffin embedded tissues are not as flexible. The fixation of tissues in formaldehyde leads to extensive crosslinking of all tissue components resulting in nucleic acid fragmentation (Lehmann and Kreipe 2001). The processing steps involved in paraffin embedding result in poor DNA integrity making it difficult to amplify by both conventional PCR and real-time PCR. Ideally it would be beneficial to apply real-time PCR to alcohol-fixed tissue samples and paraffin-embedded samples, however paraffin embedded tissues remain problematic when subjected to real-time amplification. Further studies into alternative fixation and processing steps could aid in the application of real-time PCR to paraffin embedded tissue samples, followed by the establishment of methods for pathogen control.

SYBR Green I is used in real-time PCR methods because it is cheap, simple to use and the results it generates are easy to interpret (Giglio et al. 2003). The disadvantage of SYBR Green is that it binds to any double stranded DNA and can result in binding to non-specific PCR products and primer dimers (Reece 2004). Good primer specificity is required when using SYBR Green I reducing these non-specific effects. Hybridisation probes are often employed when real-time PCR cannot be achieved using SYBR Green. Two sequence specific oligonucleotide probes labelled with different dyes bind to the target sequence in the PCR reaction. Hybridisation probes may confer greater specificity with real-time PCR and may be more effective when dealing with paraffin-embedded

samples. However SYBR Green is inexpensive making it more feasible for implementation into diagnostic laboratories.

Disease outbreaks in molluscan aquaculture can severely affect a country's aquaculture production and trade (Berthe et al. 1999). Nucleic acid-based techniques offer many advantages for aquaculture and should be routinely employed in disease diagnosis. PCR offers the advantages of rapid and specific detection of pathogens in aquatic hosts and quantitation of pathogen load has the potential to identify early and advanced pathogenic diseases in shellfish aquaculture, which is why real-time PCR is becoming such an important tool in pathology. In 2003, Bower outlined that because so few diagnostic tools were available for the detection of abalone diseases, standard histological procedures had to be used. DNA technology has been revolutionary and is the way forward in all biological disciplines including marine biology. Molecular diagnostics need to be made universally acceptable and available to marine pathologists if the causes of disease outbreaks and the presence of pathogens are to be identified and methods of control established.

CHAPTER 5

GENERAL DISCUSSION

GENERAL DISCUSSION

Sustainability has become a global phenomenon which emphasises the development of natural-resource based industries in such a manner that is compliant with the conservation of the natural environment. According to the Bruntland Commission Report (1997) 'sustainable development is development that meets the needs of the present without compromising the ability of future generations to meet their own needs'. In marine research the emphasis is on sustainable aquaculture (Meldon 1993; FAO 2004; Bell et al. 2006). Ideally sustainable aquaculture should lead to reduced pressure on natural marine stocks and should also have no adverse effects upon the surrounding natural aquatic resources. It is this type of aquaculture that has become a high priority objective in most countries.

Aquaculture is one of the fastest expanding sectors of food production in the world (Bostock 2002). Therefore, it is not surprising that global aquaculture of premium priced luxury products such as abalone, has become a thriving and profitable industry. According to Gordon and Cook (2001) worldwide catch from abalone fisheries declined by 30% from 1991-2001, however this was offset by a staggering 600% increase in the production of cultured abalone. The success of abalone aquaculture to date is overwhelming, so much so that this marine organism has been introduced as a foreign species to Ireland. Abalone are farmed on a small scale in Ireland as the costs of production are reasonably high, not to mention the long grow-out period of three years (Watson and Stokes 2003a). Despite these demands Irish abalone aquaculture has been very successful since its establishment, as the market for these shellfish is undersupplied, making it a substantially profitable industry (Watson and Stokes 2003a).

It is thus reasonable to suggest that a greater knowledge of the fundamental biology and pathogens of abalone shellfish would be beneficial to the development and expansion of abalone aquaculture. Being a relatively new venture in Ireland, there is little research being conducted into the study of these non-native marine organisms.

This project is divided into three main sections. The first two sections can be described as a histological characterisation of abalone shellfish employing the use of two techniques, firstly immunohistochemistry to examine protein distribution and secondly histochemistry to analyse the distribution of enzymes, carbohydrates, lipids, pigments and minerals in abalone tissues. The third section of this project investigated pathogens which cause potentially debilitating diseases that could impact upon Irish abalone aquaculture, if accidentally introduced through shellfish transfer and translocation. The focus of this section was on the development of nucleic acid-based methods for the detection and quantitation of abalone pathogens.

The first section of this research project was to investigate and analyse structural and functional proteins in two species of abalone, *Haliotis tuberculata* and *Haliotis discus hannai* using immunohistochemistry. There are no commercial antibodies available to abalone antigens so a panel of antibodies that react with human antigens were used. In our study three mouse antibodies to human antigens (Table 2.1) reacted positively with antigens in abalone tissues (PCNA, cytokeratin MNF and NSE). In the second panel of antibodies (with a wider range of cross reactivity) tested (Table 2.2), four mouse/rabbit antibodies to vertebrate/invertebrate antigens cross reacted with antigens in abalone tissues (PCNA, cytokeratin AE1, laminin and vimentin). Western blotting was employed to further confirm that these proteins were present in abalone tissues. Western

blotting with five antibodies revealed immunopositive bands with abalone tissues, confirming the presence of PCNA (2 antibodies), cytokeratin MNF, cytokeratin AE1 and vimentin. No significant differences were observed between the two species with immunohistochemical staining but western blot revealed different banding patterns between *H. discus hannai* and *H. tuberculata* with PCNA, cytokeratin MNF and vimentin antibodies.

Protein typing by immunohistochemistry in this study revealed the presence of structural, proliferative and functional proteins in abalone tissues. This is the first study to identify these proteins in abalone shellfish and the results obtained in this study compare well to, and are corroborated by, other studies that identified these proteins in other species. Cytokeratins are ubiquitous structural filamentous components that have been identified in the tissues of most vertebrate and invertebrate species (Karabinos et al. 1988; Markl and Franke 1988; Markl et al. 1989; Bunton 1993; Diogo et al. 1994; Lyons-Alcantara et al. 1999). Proliferative proteins involved in cell division and growth such as PCNA have been identified in many organisms (Suzuka et al. 1989; Ortego et al. 1994; Lyons-Alcantara et al. 1999) and vimentin is a common intermediate filament in invertebrate species (Walter and Biessman 1984; Karabinos et al. 1998). Laminin is a structural protein that is well conserved through species and has been detected in invertebrates (Sarras et al. 1994; Zhang et al. 1994; Yurchenco and Wadsworth 2004) and NSE is a neural isoenzyme that has been reported in invertebrates (Vullings et al. 1989). All of these proteins were detected in abalone by immunohistochemistry and western blot however NSE and laminin were not confirmed by western blot analysis. Through the identification and characterisation of these cellular proteins, immunohistochemistry may lead to the development of shellfish cell lines, as cells

could be isolated and subsequently grown in culture. According to Sahul-Hameed et al. 2006, cell lines provide an important tool for monitoring disease and studying toxicology, carcinogenesis, cellular physiology, genetic regulation and expression. Crustacean and fish cell lines have been developed (Chen et al. 1986; Hu 1990; Acosta et al. 2006; Sahul-Hameed et al. 2006) which indicates the potential for the development of shellfish and abalone cell lines as aids to the study of pathogens and pollutants.

While studies have been conducted on the identification of structural proteins in invertebrates, fish and other shellfish (Markl et al. 1989; Bunton 1993; Sevala et al. 1993; Diogo et al. 1994; Inoue et al. 1995), the proteins researched in the present study have not been previously characterised by immunohistochemistry or western blots in abalone tissues. Western blotting revealed interesting results with PCNA, cytokeratin MNF and vimentin, as specific bands other than the initial targets were identified. These findings indicate that the antibodies used were specific for other proteins that may have a slightly different conformation in abalone shellfish. The identification of proteins in tissues is an important feature of the biology and pathology of any organism and this study has provided some information about proteins in abalone, however there are multiple other proteins that have yet to be characterised in the Haliotidae, due to the lack of appropriate antibodies.

The second aim of this research project was to use histochemical techniques to evaluate the distribution of important biochemical elements in abalone tissues such as enzymes, carbohydrates, lipids, pigments and minerals. Understanding how an organism operates is a key component to the success of any culture facility and the study of these

components gives an indication of the molecular activities ongoing within cells and tissues. They function in a range of activities such as nutrition, digestion, sexual differentiation, locomotion and as indicators of environmental toxicity (Voltzow 1994; Carballal et al. 1997; Mikhailov et al. 1997; Galloway et al. 2002; García-Carreño et al. 2003; Bonacci et al. 2004). Research into such components provides invaluable information for shellfish growers, as they can utilise the information generated to formulate specialised feeds and design diets which increase the efficiency of shellfish growth (Troell et al. 2006), which in turn maximises efficiency and profitability.

The primary aim of this section of the research was to create a complete and comprehensive analysis of the various cell components and molecular substances present in abalone tissues (*Haliotis tuberculata* and *Haliotis discus hannai*). The tissues in each organ of abalone were systematically assessed for the presence or absence of enzymes, carbohydrates, lipids, pigments and minerals using a panel of histochemical stains. No significant differences were observed between these two species as similar staining patterns for enzymes, carbohydrates and lipids were obtained for each species. Pigment and mineral analyses differed slightly between the two species as calcium and copper were only identified in one *H. discus hannai* sample. While these components have been researched in molluscs, a histochemical analysis of these tissue entities in the Haliotidae has not been published.

Enzyme activities in molluscs have been investigated as regards their roles in digestion, immune response and as biomarkers of pollution (Carballal et al. 1997; Mora et al. 1999; García-Carreño et al. 2003). Most studies rely on enzyme extraction and other techniques and few employ histochemistry as a tool to analyse enzyme distribution

(Serviere-Zaragoza 1997; Hernandez-Santoyo et al. 1998; Picos-García 2000). Results from this study revealed the presence of enzymes including esterases, phosphatases, peroxidases, cholinesterases and peptidases in abalone tissues using enzyme histochemistry. Non-specific esterases, phosphatases, peptidases and peroxidases have been reported in many shellfish species such as the Pacific oyster *Crassostrea gigas,* the Spanish mussel *Mytilus galloprovincialis* and the Asiatic clam *Corbicula fluminea,* the green abalone *Haliotis fulgens* and the bivalve mollusc *Pinctada fucata* (Mora et al. 1999; Picos-García 2000; Xiao et al. 2002; Luna-González et al. 2004). Enzyme activity is an important aspect of abalone physiology and each organ enlists different enzymes to perform specific functions. Digestive enzymes are foremost in molluscan research as a result of their implications for successful aquaculture; however enzymes are indicators of many other important issues such as disease or environmental toxicity which could also have ramifications for culture facilities, once again signifying the possibility of further investigations into the basic biological mechanisms of abalone shellfish.

Carbohydrates (glycogen and mucins) were also analysed in abalone tissues. While glycogen is a major contributor to nutrition and energy reserves, mucins appear to play a more significant role in the form and function of abalone shellfish, as mucins were ubiquitous throughout the tissues of this organism. More specifically, mucins are mass produced by the foot and hypobranchial gland, which has been previously reported by other studies (Grenon and Walker 1978; Bolognani-Fantin and Ottaviani 1981). This was confirmed by our study and, in addition, mucin distribution by type was further analysed (neutral, acidic, sulphated and strongly sulphated mucins) which has not been previously reported. It is suggested that the energy metabolism of abalone is based upon carbohydrates as their natural diet is rich in carbohydrate, so their study is an important

aspect of abalone aquaculture through the development of effective formulated diets for optimal abalone growth (Du and Mai 2004).

A study of lipids, pigments and minerals in abalone tissues was also undertaken in this research project. Lipid histochemistry is not a widely used technique but it successfully identified neutral lipids in the female gonad and digestive gland of *Haliotis* spp. Lipids are important components in the maintenance of eggs and larvae (Moran and Manahan 2003) and are involved in the provision of energy for digestion (Mai et al. 1995; Gordon et al. 2006). However there still remains some debate as regards the effect that high lipid concentration can have on abalone growth (Moran and Manahan 2003; Thongrod et al. 2003; Viera et al. 2005).

A selection of pigments and minerals were examined as part of this study. Iron and copper were identified in the digestive gland and hypobranchial gland respectively, indicating their accumulation from the external environment. Previous studies have reported similar findings of metals residing in the internal organs of molluscs (Bryan et al. 1977; Bevelander 1988; Carefoot et al. 2000). Monitoring of heavy metal accumulation is essential as the effects of these pollutants become more severe as concentrations become greater (Gaetke and Chow 2003; Hernández et al. 2006) Analyses such as these provide essential information on environmental quality which highlights the advantages of these methods and these molluscs as potential biomarkers for pollution and water quality. Seasonality is an important aspect of molluscan physiology as the levels of heavy metals or other bioindicators will fluctuate throughout the year depending upon factors such as diet and nutrient availability, reproductive status, disease and other factors (Sheehan and Power 1999). Calcium and melanin were

also identified in this study and are not externally acquired entities, rather they are produced internally by the organism and have some physiological role or are merely by-products of metabolism.

The key to understanding the biology of any organism requires a fine knowledge of tissues and cells which is what the first two, predominantly histological, sections of this study planned to achieve. Based on these findings a more detailed and comprehensive database on the physiological and biochemical mechanisms of the *Haliotis* spp. has been created which has potential for use in the development of shellfish cell lines, the development of formulated diets in aquaculture, in environmental assessment and in the study of disease and methods of control.

In the final section of this study the aim was to identify abalone pathogens of importance and develop molecular methods for their detection. In view of the global spread of infectious disease in shellfish culture facilities, the detrimental effects upon shellfish stocks and the preferred absence of disease from small-scale Irish abalone culture facilities, rapid pathogen detection is important. Current traditional pathogen testing methods have limitations, so the development of more modern nucleic acid-based technology is crucial. Molecular biology has been a revolutionary development allowing for the ultimate progression from conventional biology to the exploration of the genetic and biochemical mechanisms that govern biological processes. The absence of molecular diagnostics from diagnostic laboratories indicates the need for the adoption and validation of these techniques, which offer significant advantages over traditional histological screening methodologies (Cunningham 2002; OIE 2003).

In this section of the study two major pathogens of abalone were chosen for analysis. Bacteria and protozoa are the most commonly encountered pathogens in abalone shellfish. *Vibrio harveyi* is a highly pathogenic bacterium to abalone and *Perkinsus olseni* is a protozoan that also causes severe disease in this shellfish. *Vibrio harveyi* and *Perkinsus olseni* are ranked as Category I pathogens that have been recognised as causing serious mortality in both wild and cultured abalone (Bower 2000).

In this study a novel multiplex PCR method was developed to simultaneously amplify a 413 bp region of the 16S rRNA sequence of *Vibrio harveyi* and a 155 bp region of the actin mRNA gene sequence of *Haliotis* spp. This multiplex PCR was used to amplify these sequences in both fixed tissues and paraffin embedded tissues of infected *Haliotis tuberculata*. Novel primers were designed to target a 265 bp region of the ITS sequence of *Perkinsus olseni* from paraffin embedded samples of infected *Ruditapes decussatus*, which could be adapted to detect *Perkinsus olseni* in paraffin embedded abalone tissues.

PCR was used as a method of detection in this study as it is sensitive, specific and can be completed within a relatively short period of time. Multiplex PCR is specific and allows for amplification of multiple targets in a single PCR reaction (Rodkhum 2006). It was developed in this study to include an internal control in the PCR reaction, as DNA extracted from paraffin-embedded tissues is often of poor quality (Lehmann and Kreipe 2001). Thus the inclusion of an internal control such as a housekeeping gene will give a good indication of the integrity of the extracted DNA and prevent the occurrence of false negative test results, which is an important element of pathogen testing in aquaculture facilities.

LightCycler® real-time quantitative PCR was also developed in this study for the detection of *Vibrio harveyi* and *Perkinsus olseni*. Real-time SYBR Green PCR was chosen as a method of detection as it is specific, easy to use and rapid results are generated (Mackay 2004; Vadopalas et al. 2006). The closed single tube assay eliminates possible contamination, does not require post-PCR analysis such as gel electrophoresis and most advantageously allows for quantitation of pathogen load. The *Vibrio harveyi* real-time PCR method can detect copy numbers of 10^3 and the *Perkinsus olseni* real-time PCR method can successfully detect copy numbers of 10^2. From the time DNA is extracted from samples to application of standard PCR testing, a positive diagnosis can be generated in seven hours. With real-time PCR, a diagnosis, be it positive or negative, can be made in less than three hours. These methods are being developed for pathogen detection in the medical, agriculture and marine industries and will be major diagnostic methods in future years.

The advantages of nucleic acid-based techniques over histological methods for pathogen detection remain unrivalled. Even in the molecular area there are many new techniques that are challenging standard PCR detection methods such as gene chip/microarray methods which allow for the monitoring of expression levels for thousands of genes simultaneously. They are used to compare gene expression in diseased and normal cells and can be used for rapid analysis. This study shows that real-time PCR is an easy method to establish, but further optimisation for application to paraffin embedded samples is required for the accurate detection and quantitation of pathogen load. More specific detection methods such as real-time PCR using hybridisation probes may be more effective but there are the associated costs involved, which renders SYBR Green methods more suitable for application to diagnostic laboratories. The methods described

in this study allow for rapid pathogen detection and quantitation of pathogen load, indicating the potential of these nucleic-acid based methods for future diagnostic application and their use in routine screening.

Molecular species identification techniques have a broad range of applications in the management and conservation of marine organisms (Sweijd et al. 2000). Incorporation of molecular nucleic acid-based techniques into marine diagnostic labs would aid in epidemiological studies as well as identifying the causes of disease outbreaks or the presence of pathogens, but most importantly, it would greatly increase the speed of disease diagnosis (Cunningham 2002). Recently developed nucleic acid-based techniques for the detection of pathogens such as *Perkinsus* spp. and *Vibrio* spp. are indicative of the significant advantages of these methods (Audemard et al. 2004; Elandalloussi et al. 2004; Panicker et al. 2004). The main developments currently in R&D (research and development) for effective disease management in shellfish farms are the development of rapid, sensitive, cost-effective and convenient methods for the detection of shellfish pathogens and the development of disease resistant shellfish strains (Bostock 2002).

At present, restriction enzyme digestion, probe hybridisation and polymerase chain reaction are the techniques most commonly applied in the routine diagnosis of fish diseases and in some cases of shellfish diseases (Cunningham 2002). However more innovative nucleic acid-based methods are being developed and if implemented they present the opportunity for marine research to enter a new realm of molecular technology. DNA markers facilitate rapid progress in aquaculture investigations of genetic variability and inbreeding, parentage assignments, species and strain

identification and the construction of high resolution genetic linkage maps for aquaculture species (Liu and Cordes 2004). Molecular techniques such as gene transfer allow for the development of transgenic animals which provide a powerful system for the *in vivo* study of gene regulation, expression and function (Chen et al. 2006). Electroporation of exogenous DNA into fertilised eggs has been conducted (Inoue et al. 1990; Powers et al. 1995) but direct microinjection is a one-shot, minimally invasive and high throughput technique for DNA transfer (Chen et al. 2006). Chen et al. 2006 have recently experimented with gene transfer in Japanese abalone. They used direct microinjection methods to transfer exogenous DNA fragments (growth hormone DNA) into the testis of abalone in an attempt to achieve faster growth. Southern blot analysis revealed that the transgene was integrated into the genome of experimental abalone indicating the simplicity and efficiency of this method for the production of transgenic abalone. This also has potential for application in the development of disease resistant animals. Techniques such as arbitrary fragment length polymorphism (AFLP) are also being used to identify genetic markers associated with resistance to *Vibrio* spp. and *Perkinsus* spp. in eastern oysters, which promotes the development of selective breeding programmes and reduces the risk of mass mortalities caused by these pathogens (Sokolova et al. 2006).

With this project an in-depth biological study of *Haliotis* spp. was performed to analyse its gross morphology, histology and pathology. It has identified the distribution of various cell components including enzymes, carbohydrates, lipids, pigments, minerals and proteins which are all involved in the organism's functional biological processes. In an effort to detect and potentially control disease outbreaks, nucleic acid-based detection methods were developed that successfully detect abalone pathogens. It

provides two early detection systems for harmful shellfish pathogens and provides a basic biological reference system which presents opportunities for further research in this area. The body of research presented here will prove useful for marine scientists and shellfish growers alike, in this potential multi-billion euro Economic Zone.

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APPENDICES

APPENDIX A: CHAPTER 2 SOLUTIONS

DAVIDSON'S FIXATIVE

200 ml Glycerol

400 ml 37-40% Formaldehyde

600 ml 95% ETOH

200 ml Acetic Acid

600 ml Sea-water

PBS

1 PBS (Oxoid) tablet was dissolved in 100 ml distilled water

COMPLETE RPMI 1640 MEDIUM (Sigma-Aldrich)

500 ml RPMI 1640

5 ml 2 mM Glutamine

50 ml 10% Foetal Calf Serum (FCS)

2 ml PenStrep antibiotics (10,000 units Penicillin: 10 mg/ml Streptomycin in 0.9% NaCl).

TRYPSIN (25 g/l porcine trypsin in Hank's Balanced Salt Solution with Phenol red)

50 ml 2.5% Trypsin (Sigma-Aldrich)

500 ml Earle's balanced salts

0.02% VERSENE (Autoclaved)

0.02 g EDTA

100 ml PBS

SDS LYSIS BUFFER

2% SDS

50 mM Tris-HCl (pH 7.2)

1 mM β-mercaptoethanol

NP40 TRIPLE DETERGENT LYSIS BUFFER

0.1% SDS

150 mM NaCl

50 mM Tris.Cl (pH 8.0)

1% Nonidet P-40 (NP-40)

12% RESOLVING GEL

3.3 ml distilled H_2O

4 ml 30% Acrylamide mix (40:1)

2.5 ml 1.5 M Tris.Cl pH 8.8

0.1 ml 10% SDS

0.1 ml 10% Ammonium persulphate

5 µl TEMED

STACKING GEL

2.1 ml distilled H_2O

0.5 ml 30% Acrylamide mix (40:1)

0.38 ml 1 M Tris.Cl pH 6.8

30 µl 10% SDS

30 µl 10% Ammonium persulphate

3 µl TEMED

1X RUNNING BUFFER

6.1 g Tris

28.8 g Glycine

2 g SDS

Tris, glycine and SDS were dissolved in 1900 ml distilled water and the pH was adjusted to 8.3 with 1 M NaOH. The solution was made up to 2 L with distilled water in a volumetric flask.

2X SDS LOADING BUFFER

10 ml Glycerol

5 ml β-mercaptoethanol

2 g SDS

0.756 g Tris

0.001 g Bromophenol blue

SDS and Tris were weighed out in 30 ml distilled water. Glycerol, β-mercaptoethanol, and bromophenol blue were added and made up to 50 ml with distilled water in a volumetric flask. Stored as 1 ml aliquots at 4°C.

TRANSFER BUFFER

3.03 g Tris

14.42 g glycine

200 ml methanol

600 ml distilled water

TBS BUFFER (pH 7.4)

6.05 g Tris

8.766 g NaCl

800 ml distilled water

The pH was adjusted to 7.4 and made up to 1 L in a volumetric flask.

5% BSA BLOCKING SOLUTION

5g BSA

100 ml TBS

APPENDIX B: CHAPTER 2 POSITIVE CONTROLS

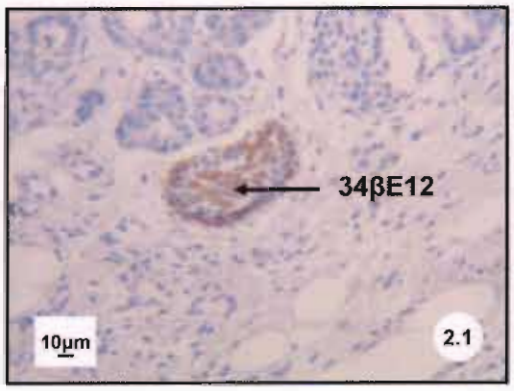

Figure 2.1: Positive staining of pancreas control with 34βE12 (x400 digital camera).

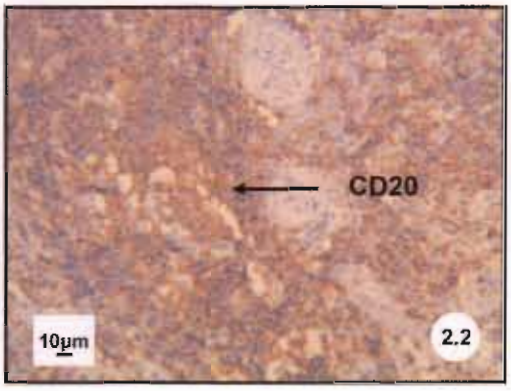

Figure 2.2: Positive staining of tonsil control with CD 20 (x400 digital camera).

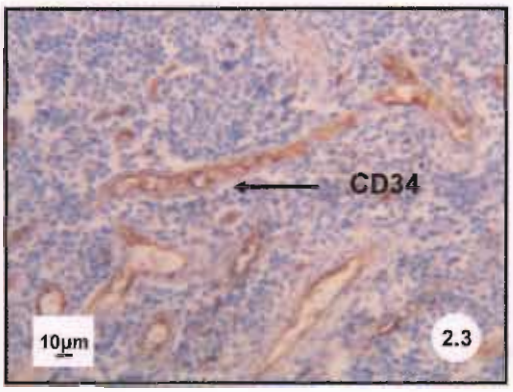

Figure 2.3: Positive staining of tonsil control with CD 34 (x400 digital camera).

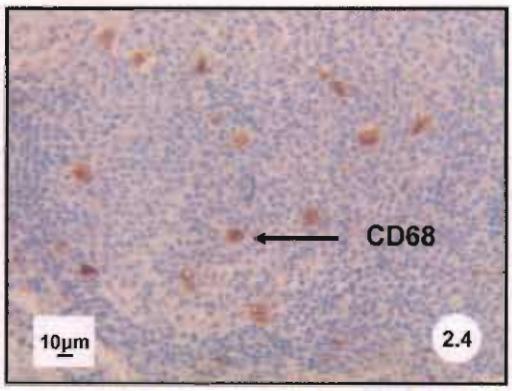

Figure 2.4: Positive staining of tonsil control with CD 68 (x400 digital camera).

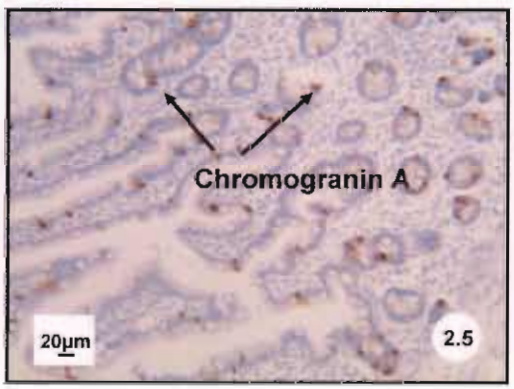

Figure 2.5: Positive staining of ileum control with Chromogranin A (x200 digital camera).

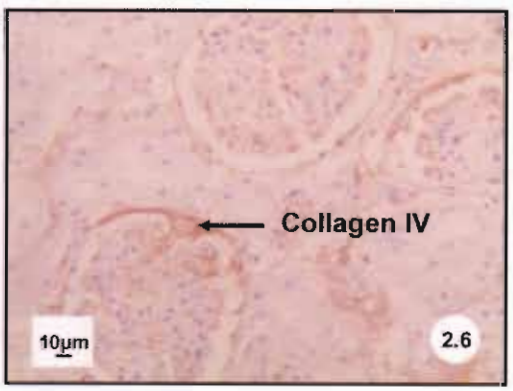

Figure 2.6: Positive staining of kidney control with collagen IV (x400 digital camera).

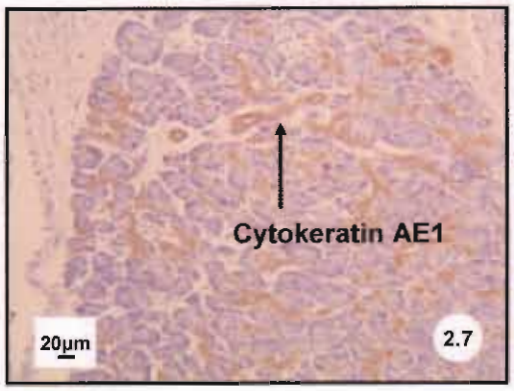

Figure 2.7: Positive staining of pancreas control with cytokeratin AE1 (x200 digital camera).

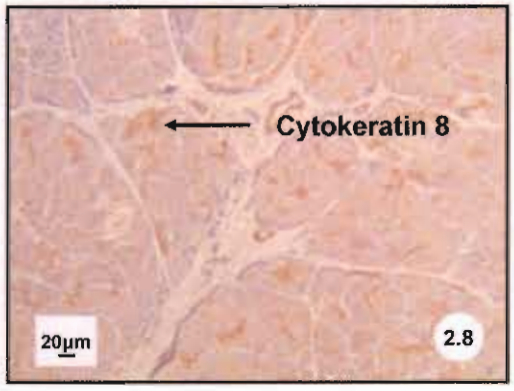

Figure 2.8: Positive staining of pancreas control with cytokeratin 8 (x200 digital camera).

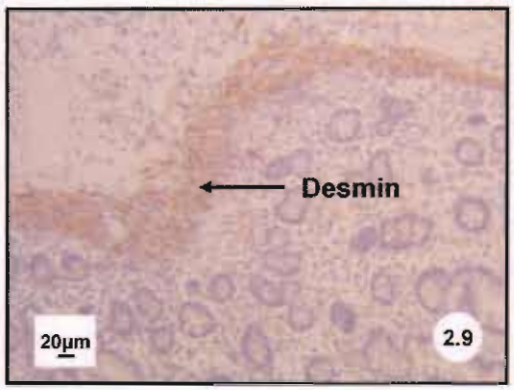

Figure 2.9: Positive staining of intestine control with desmin (x200 digital camera).

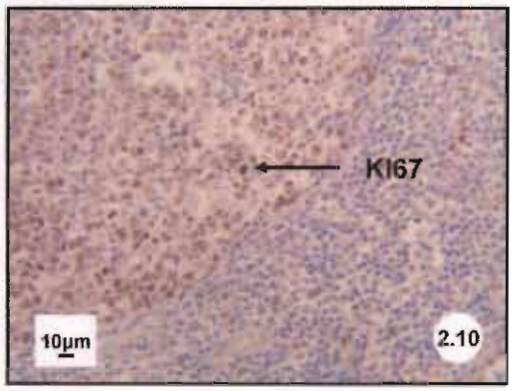

Figure 2.10: Positive staining of tonsil control with Ki67 (x400 digital camera).

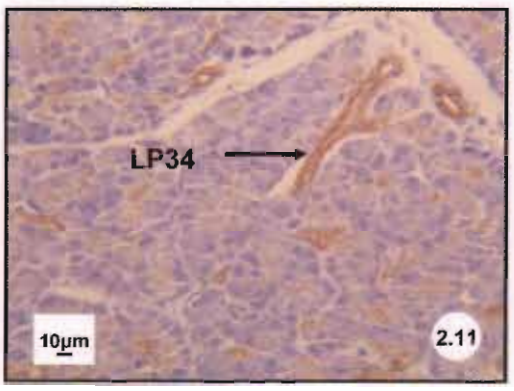

Figure 2.11: Positive staining of skin control with LP34 (x400 digital camera).

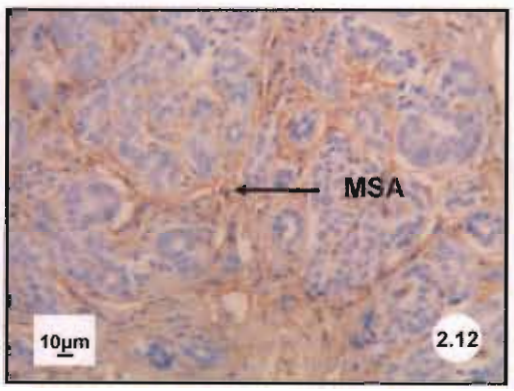

Figure 2.12: Positive staining of pancreas control with MSA (x400 digital camera).

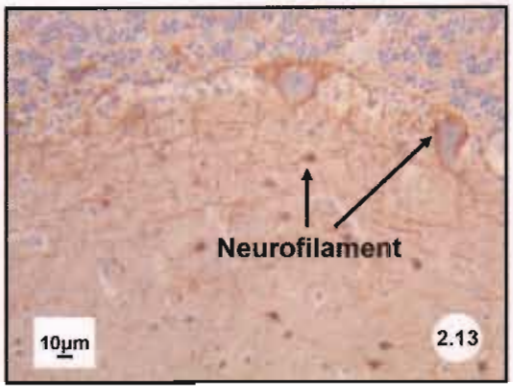

Figure 2.13: Positive staining of cerebellum control with neurofilament (x400 digital camera).

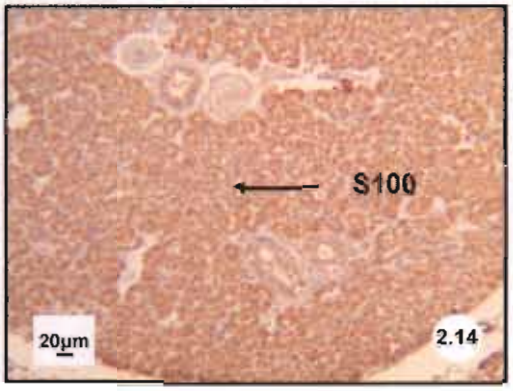

Figure 2.14: Positive staining of salivary gland control with S100 (x200 digital camera).

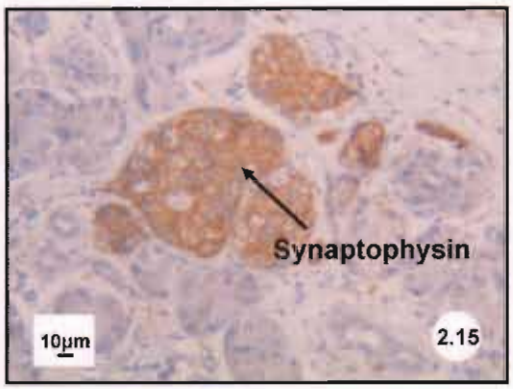

Figure 2.15: Positive staining of pancreas control with synaptophysin (x400 digital camera).

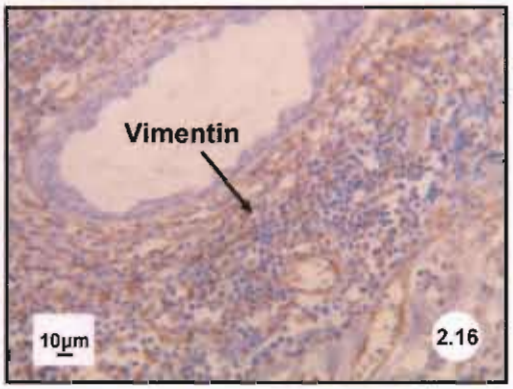

Figure 2.16: Positive staining of tonsil control with vimentin (x400 digital camera).

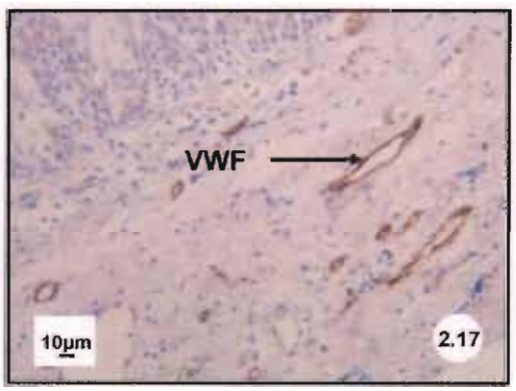

Figure 2.17: Positive staining of tonsil control with VWF (x400 digital camera).

APPENDIX C: CHAPTER 3 STAINING PROTOCOLS AND SOLUTIONS

HAEMATOXYLIN AND EOSIN

1. Sections were dewaxed to water.
2. Sections were stained in Harris' Haematoxylin. 5 min
3. Sections were differentiated in 1% acid-alcohol. 2 sec
4. Sections were blued in running (warm) tap water to raise the pH and change the colour of the nuclei from red to blue/black. 5 min
5. Sections were checked microscopically: background should be clear and nuclei should be blue.
6. Sections were stained in 1% Eosin. 2 min
7. Slides were washed in water.
8. Sections were dehydrated: spirit (3 dips), absolute alcohol (3min), absolute alcohol (6 min), xylene (5 min), xylene (5 min).
9. Sections were mounted in DPX.

Nuclei:	**Blue/purple**
Cytoplasm/Fibres:	**Red/pink**

ACID PHOSPHATASE (Catalogue No. 387 Sigma-Aldrich)

Reagents:

Fast Garnet GBC Base Solution: Fast garnet GBC base, 7 mg/ml, in 0.4 mol/l hydrochloric acid with stabilizer.

Sodium Nitrite Solution: Sodium Nitrite, 0.1 mol/l.

Napthol AS-BI Phosphoric Acid Solution: Napthol AS-BI phosphoric acid, 12.5 mg/ml.

Acetate Solution: Acetate buffer, 2.5 mol/l, pH 5.2 ± 0.1.

Mayer's Haematoxylin.

1. Sufficient distilled water was warmed to 37°C.
2. To each of two test tubes, 0.5 ml Fast Garnet GBC Base Solution and 0.5 ml sodium nitrite solution was added. They were mixed gently by inversion for 30 sec and allowed to stand for 2 min.
3. Into two 100 ml beakers A and B the following were added while mixing:

	Beaker A	**Beaker B**
Distilled water warmed to 37°C	45 ml	45 ml
Diazotized Fast Garnet GBC Solution	1 ml	1 ml
Naphthol AS-BI Phosphate Solution	0.5 ml	0.5 ml
Acetate Solution	2 ml	2 ml

1. Formalin fixed sections were incubated for 1 h at 37°C.
2. Slides were rinsed in distilled water.
3. Sections were counterstained in Mayer's haematoxylin for 1 min.
4. Slides were rinsed and sections were mounted in warm glycerine jelly.

Enzyme Activity: **Pink**

ALKALINE PHOSPHATASE (Catalogue No. 86 Sigma-Aldrich)

Reagents:

Sodium Nitrite Solution: Sodium nitrite, 0.1 mol/l.

FRV Alkaline Solution: Fast red violet LB base, 5 mg/ml, in hydrochloric acid, 0.4 mol/l, with stabiliser.

Naphthol AS-BI Alkaline Solution: Naphthol AS-BI Phosphate, 4 mg/ml, in AMPD buffer, 2 mol/l, pH 9.5.

Mayer's Haematoxylin.

1. 45 ml of distilled water was incubated at 18-25°C.
2. The diazonium salt solution was prepared by adding 1 ml Sodium Nitrite Solution to 1 ml of FRV-Alkaline Solution.
3. Diazonium Salt Solution was added to the distilled water from step 1.
4. 1 ml Naphthol AS-BI Alkaline Solution was added to diluted diazonium salt solution, mixed thoroughly and poured into a coplin jar.
5. Formalin fixed sections were added to the alkaline dye mixture and incubated at 18-26°C for 15 min. Slides were protected from direct light.
6. After 15 min incubation the slides were rinsed in distilled water.
7. Sections were counterstained in Mayer's Haematoxylin for 40 sec - 1 min.
8. Sections in were mounted in warm glycerine jelly.

Enzyme Activity: **Pink**

ALPHA NAPHTHYL BUTYRATE ESTERASE (Catalogue No. 181-B Sigma-Aldrich)

Reagents:

Alpha Naphthyl Butyrate Solution: alpha-naphthyl butyrate, 2.4 g/L, in methanol solution with solubilizers.

Pararosaniline Solution: Pararosaniline, 40 g/L, in 2 mol/L hydrochloric acid.

Sodium Nitrite Tablets: sodium nitrite, 0.1 molecular/L.

Phosphate Buffer: Sodium and potassium phosphates.

Mayer's Haematoxylin.

Citrate Solution.

1. Phosphate buffer solution was warmed to 37°C.
2. 1.5 ml sodium nitrite tablet solution was added to 1.5 ml pararosaniline solution.
3. They were mixed gently by inversion and left to stand for 5 min then added to 40 ml of prewarmed phosphate buffer.
4. 5 ml of alpha naphthyl butyrate esterase solution was added.
5. They were mixed well and poured into a coplin jar. The solution turned amber in colour.
6. Formalin fixed sections were incubated in solution at 37°C for 1 h.
7. Slides were rinsed in running tap water.
8. Slides were left to air dry for 15 min prior to counterstaining.
9. Sections were counterstained with Mayer's Haematoxylin for 1 min.
10. Sections were coverslipped in glycerine jelly.

Esterase activity: **Red**

NAPHTHOL AS-D CHLOROACETATE ESTERASE (Catalogue No. 91-C Sigma-Aldrich)

Reagents:

Sodium Nitrite Solution: Sodium nitrite, 0.2 mol/L.

Fast Red Violet LB Base Solution: Fast red violet LB base, 15 mg/ml, in 0.4 mol/L hydrochloric acid with stabilizer.

TRIZMAL™ pH 6.3 Buffer Concentrate: TRIZMA® maleate, 1 mol/L, with surfactant, pH 6.3 @ 25°C.

Naphthol AS-D Chloroacetate Solution: Naphthol AS-D chloroacetate, 8 mg/ml and stabilizer.

Mayer's Haematoxylin.

1. Sufficient distilled water was warmed to 37°C.
2. 1 ml sodium nitrite solution was added to 1 ml fast red violet LB base solution. Solutions were mixed gently by inversion and left to stand for 2 min.
3. The solution from step 2 was added to 40 ml warmed distilled water.
4. 5 ml TRIZMAL pH 6.3 buffer concentrate was added.
5. 1 ml naphthol AS-D chloroacetate solution was added and this mixture turned red.
6. Fomalin fixed sections were incubated for 30 minutes at 37°C.
7. Slides were rinsed in distilled water.
8. Sections were counterstained in Mayer's Haematoxylin for 2 min.
9. Slides were washed in running water for 5 minutes and sections were mounted in glycerine jelly.

Esterase activity: **Red**

HANSSON'S CARBONIC ANHYDRASE

Solution A: To 1 ml of 0.1 M $CoSO_4$ (cobalt sulphate), 0.05 M H_2SO_4 (sulphuric acid) was added.

Solution B: 1 g $NaHCO_3$ (sodium hydrogen carbonate) was dissolved in 50 ml of 0.1 M Na_2SO_4 (sodium sulphate).

1. Before incubation solution B was poured into solution A in order to avoid a momentary high concentration of cobalt ions.
2. Slides were incubated for 60-120 min at RT.
3. Slides were washed in distilled water.
4. Slides were transferred to 1% $(NH_4)_2S$ (ammonium sulphide solution) for 1 min.
5. Slides were washed in water and sections were mounted in warm glycerine jelly.

CAH activity: **Black/brown deposits**

LEUCINEAMINOPEPTIDASE

Solutions:

A. Substrate Solution:

1-leucyl-4-methoxy β naphthylamide	4 mg
Ethyl alcohol	0.1 ml
dH_2O	4.9 ml

B. Sodium Chloride:

Sodium chloride	425 mg
dH_2O	50 ml

C. Copper sulfate:

Copper sulfate	798 mg
dH_2O	50 ml

D. Potassium cyanide Solution:

Potassium cyanide	65 mg
dH_2O	50 ml

E. 0.1 M acetate buffer, pH 6.5

Incubating Medium:

Solution A	**0.5 ml**
Solution E	**5 ml**
Solution B	**4 ml**
Solution D	**0.5 ml**
Fast Blue B salt	**5 mg**

1.	Slides were incubated in incubating medium.	15 min- 2h
2.	Slides were rinsed in saline solution B.	2 min
3.	Slides were immersed in copper sulfate solution C.	2 min
4.	Slides were rinsed in saline.	2 min
5.	Sections were counterstained Mayer's Haematoxylin.	1 min
6.	Slides were washed in dH_2O.	
7.	Sections were dehydrated and mounted in glycerine jelly.	

LAP activity: **Red**

Nuclei: **Blue**

ACETYLCHOLINESTERASE

Incubation Medium:

Acetylthiocholine iodide	5 mg
0.1 M acetate buffer pH 6	6.5 ml
0.1 M sodium citrate	0.5 ml
30 mmol/l copper sulfate	1 ml
dH_2O	1 ml
4 mm/l iso-octamethyl pyrophosphoramide (iso-OMPA)	0.2 ml

1 ml 5 mmol/l potassium ferricyanide is added just before use.

Method:

1.	Slides were incubated in the incubating medium @ 37°C.	1 h
2.	Slides were washed in tap water.	
3.	Sections were treated with 0.05% p-phenylene diamine dihydrochloride in 0.05M phosphate buffer pH 6.8 for 45 minutes at RT.	
4.	Slides were washed in H_2O.	
5.	Sections were treated with 1% osmium tetroxide at RT.	10 min
6.	Slides were washed in H_2O.	
7.	Sections were counterstained in Mayer's Haematoxylin and mounted in DPX.	10sec

Nerve fibres and cells containing acetylcholinesterase: **Dark brown/black**

PEROXIDASE

1. Slides were incubated in 0.06% 3,3-diaminobenzidine tetrahydrochloride (DAB) and 0.03% hydrogen peroxide. 10-15 min
2. Sections were stained in Mayer's haematoxylin. 1 min
3. Slides were coverslipped in glycerine jelly.

Peroxidase activity: **Brown deposits**

ALCIAN BLUE/PAS

1.	Sections were dewaxed to water.	
2.	Sections were treated with Alcian Blue pH 2.5 solution.	5 min
3.	Slides were washed in distilled water.	
4.	Sections were treated with 1 % periodic acid.	5 min
5.	Slides were rinsed in distilled water.	
6.	Sections were treated with Schiff's reagent.	8 min
7.	Slides were washed in running water.	10 min
8.	Slides were counterstained in Mayer's Haematoxylin.	30 sec
9.	Sections were blued in warm running water.	3 min
10.	Slides were dehydrated, cleared and sections were mounted in DPX.	

Acid mucins:	**Sky blue**
Neutral mucins and glycogen:	**Magenta**
Nuclei:	**Deep blue**

PERIODIC ACID SCHIFF (PAS)

1. Sections were dewaxed and rehydrated to water.
2. Sections were treated with 1% periodic acid. 8 min
3. Slides were rinsed in distilled water.
4. Sections were treated with Schiff's reagent. 10 min
5. Slides were washed in running tap water. 10 min
6. Sections were counterstained in Mayer's Haematoxylin. 1 min
7. Sections were blued in running water. 5 min
8. Sections were dehydrated, cleared and sections were mounted in DPX.

Glycogen and selected mucins: **Magenta**

Nuclei: **Blue**

DIASTASE PERIODIC ACID SCHIFF (DPAS)

1. 2 slides were dewaxed and rehydrated to water (Label one DPAS as glycogen negative control).
2. The DPAS section was covered section with saliva. 10 min
3. The slides were rinsed in distilled water.
4. Sections were treated with 1% Periodic acid. 8 min
5. Slides were washed in running tap water. 10 min
6. Sections were treated with Schiff's Reagent. 10 min
7. Slides were washed in running tap water. 10 min
8. Sections were counterstained in Mayer's Haematoxylin. 1 min
9. Sections were blued in running water. 5 min
10. Slides were dehydrated, cleared and sections were mounted in DPX.

Diastase treated slides were devoid of glycogen so only mucins were magenta.

Mucins: **Magenta**

Nuclei: **Blue**

MILD PERIODIC SCHIFF (PAS)

1. Slides were dewaxed and rehydrated to water.
2. Sections were treated with 0.01% periodic acid. 8 min
3. Slides were washed in distilled water.
4. Sections were treated with Schiff's reagent. 10 min
5. Slides were washed in running tap water. 10 min
6. Sections were counterstained in Mayer's Haematoxylin. 1 min
7. Sections were blued in running water. 5 min
8. Slides were dehydrated, cleared and sections were mounted in DPX.

Glycogen and selected mucins: **Magenta**

Nuclei: **Blue**

ALCIAN BLUE (pH 2.5 /0.2)

1. Slides were dewaxed to water.
2. Sections were treated with Alcian Blue pH2.5/pH0.2 solution. 5 min
3. Slides were washed in water.
4. Sections were counterstained with 1% Eosin. 1 min
5. Slides were washed, dehydrated, cleared and sections were mounted in DPX.

Acid mucins:	**Sky Blue**
Nuclei:	**Red**

METHYLATION/SAPONIFICATION TECHNIQUE

Methylation Agent:

Concentrated hydrochloric acid	0.8 ml
Methanol	99.2 ml

Saponification Agent:

Potassium hydroxide	1 g
Ethanol	70 ml
Distilled water	30 ml

1. 3 control sections and 3 test sections were dewaxed and labelled A, B and C. They were covered with a film of 0.5% celloidin.
2. A and B were placed in preheated methanol-hydrochloric acid solution for 5 h @ 60°C.
3. All sections were washed in running water for several min.
4. A was treated with saponification agent for 30 min at RT. B and C were placed in alcohol for this period of time.
5. Slides were washed in running water for 5 min, celloidin was removed by rinsing in absolute alcohol, then in equal parts of alcohol and ether. Slides were washed again in water.
6. Sections were stained in standard alcian blue solution (pH 2.5) for 5 min.
7. Slides were washed in water.
8. Sections were counterstained with 0.1 % aqueous eosin for 20-30 sec.
9. Slides were rinsed in absolute alcohol.
10. Slides were dewaxed and cleared in xylene and sections were mounted in DPX.

A. Carboxylated mucins	**Blue**
B. Sulphated and carboxylated mucins	**Negative**
C. Sulphated and carboxylated mucins	**Blue**
Background	**Pale pink**

OIL RED O

1. Frozen sections were rinsed in distilled water.
2. Slides were transferred to 60% Isopropanol. 3 min
3. Sections were stained in Oil Red O solution. 15 min
4. Slides were washed in 60% Isopropanol until the background was clear. 1 min
5. Slides were washed in water.
6. Sections were stained in Mayer's Haematoxylin. 40 sec
7. Sections were blued in warm water. 3 min
8. Sections were mounted in glycerine jelly (60°C from oven).

Nuclei: **Blue**

Neutral Lipids and Phospholipids: **Red**

Solutions

Oil Red O STOCK Solution

Oil Red O saturated solution in 99% Isopropanol.

Oil Red O: 0.5 g

99% Isopropanol: 100 ml

Oil Red O Solution

This was prepared one hour in advance by mixing three parts of Oil Red O stock solution with two parts distilled water and filtered just before use.

RHODANINE STAIN

1. Sections were dewaxed to water.
2. Slides were incubated in rhodanine working solution at 56°C. 3 h
3. Slides were washed in several changes of distilled water.
4. Sections were stained with Mayer's Haematoxylin. 40 sec
5. Slides were rinsed in distilled water, then quickly in borax solution (0.5%) and again rinsed well in distilled water.

Copper deposits: **Bright red**

Bile: **Green**

Rhodanine Stock Solution

p-Dimethylaminobenzylidene rhodanine	0.2 g
Ethanol	100 ml

To prepare the working solution, 3 ml of stock solution (well shaken) was diluted with 47 ml distilled water.

PERL'S PRUSSIAN BLUE

1. Sections were dewaxed and rehydrated to water.
2. Perl's solution was made up and applied immediately to the slide:

2% Potassium Ferrocyanide:	3 ml	
2% Hydrochloric acid:	3 ml	20 min

3. Slides were washed well in distilled water.
4. Sections were counterstained with 1% Eosin. 30 sec
5. Slides were rinsed in water, dipped rapidly in spirit and transferred to absolute alcohol.
6. Sections were dehydrated, cleared, and sections were mounted in DPX.

Haemosiderin and Ferric salts:	**Prussian Blue**
Background:	**Red/Pink**

MASSON FONATANA

1. Sections were dewaxed to water.
2. Silver solution was prepared.
3. Sections were placed in silver solution (Coplin Jar) @ 37°C. 60 min
4. Slides were rinsed in distilled water and checked microscopically for positive staining (silver reduction to black deposit).
5. 0.1% Gold chloride was toned in. 5 min
6. Slides were rinsed with distilled water.
7. Sections were treated with 5% sodium thiosulphate (hypo). 2 min
8. Sections were counterstained with 0.2% light green. 1 min
9. Slides were rinsed, dehydrated rapidly, cleared and mounted in DPX.
10. Note: if excess green was removed in spirit, slides were rinsed in water and restained with light green.

Melanin: **Black**

Background: **Green**

Silver Solution:

To 20 ml of 10% silver nitrate, conc. ammonia was added drop by drop until the precipitate first formed, was dissolved. 20 ml of distilled water was added and placed in a coplin jar.

ALIZARIN RED S

1. Sections were dewaxed and rehydrated.
2. Sections were transferred to Alizarin red S solution. 5 min
3. Sections were blotted, and rinsed in acetone. 30 sec
4. Sections were treated with acetone-xylene (1:1 solution). 15 sec
5. Slides were rinsed in fresh xylene and sections were mounted in DPX.

Calcium deposits: **Orange-red**

Solution:

The pH of 2% Alizarin red S (aqueous) was adjusted to 4.2 with 10% ammonium hydroxide.

APPENDIX D: CHAPTER 3 POSITIVE CONTROLS

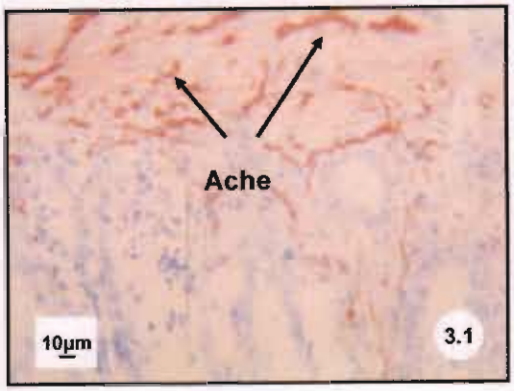

Figure 3.1: Acetylcholinesterase (brown) in Hirschprung's intestine (x400 digital camera).

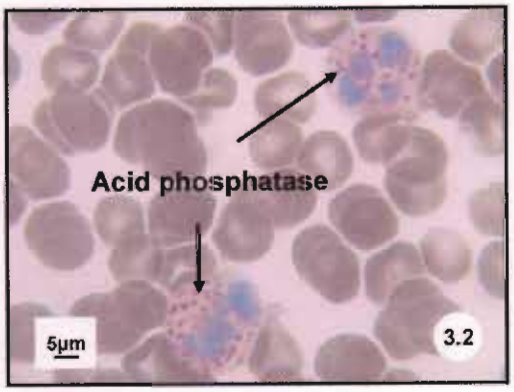

Figure 3.2: Acid phosphatase (red/brown) in a human neutrophil (x1000 oil digital camera).

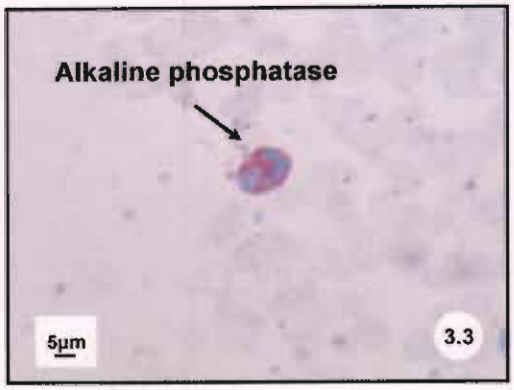

Figure 3.3: Alkaline phosphatase (pink) in a human neutrophil (x1000 oil digital camera).

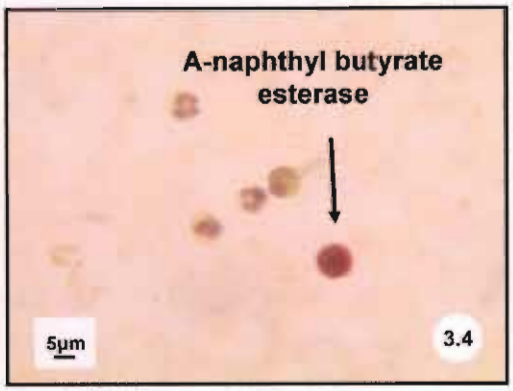

Figure 3.4: Alpha naphthyl butyrate esterase (brown/red) in a human monocyte (x1000 oil digital camera).

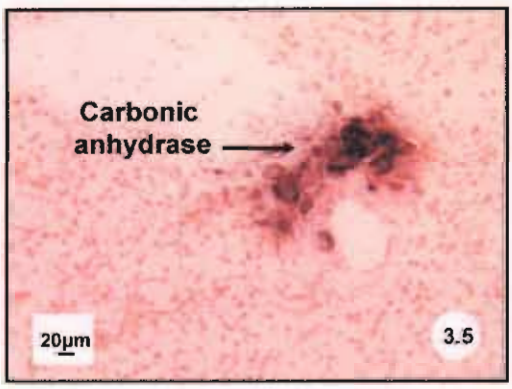

Figure 3.5: Carbonic anhydrase (black) in rat pancreas (x1000 oil digital camera).

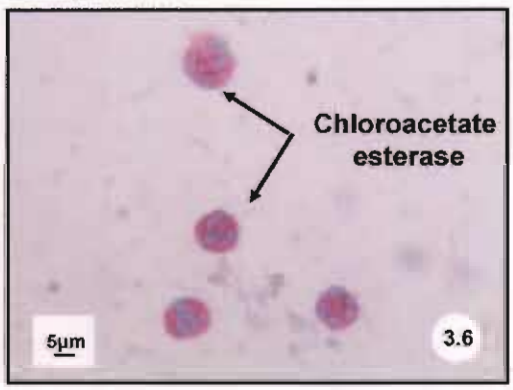

Figure 3.6: Chloroacetate esterase (pink) in a human neutrophil (x1000 oil digital camera).

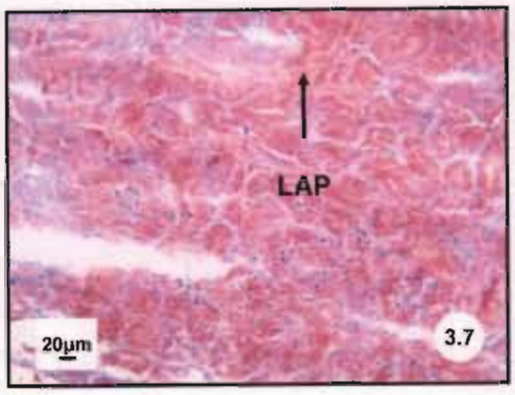

Figure 3.7: Leucineaminopeptidase (red) in rat kidney (x200 digital camera).

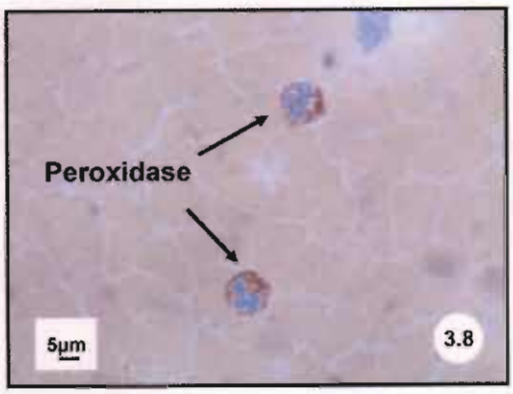

Figure 3.8: Peroxidase (brown) in a human neutrophil (x1000 oil digital camera).

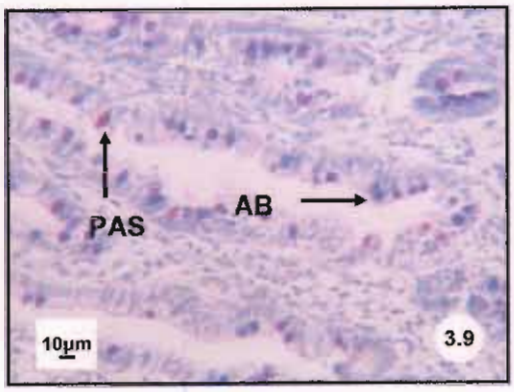

Figure 3.9: ABPAS (blue and magenta) staining of mucous cells in rat ileum (x400 digital camera).

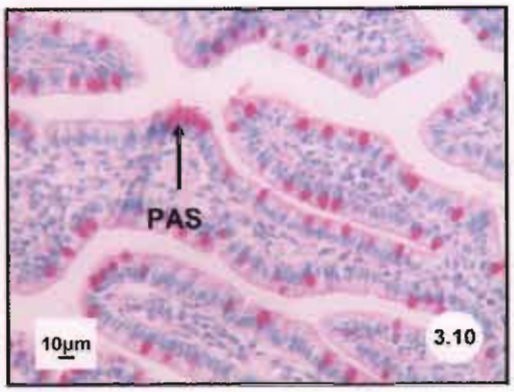

Figure 3.10: PAS (magenta) staining of mucous cells in rat ileum (x400 digital camera).

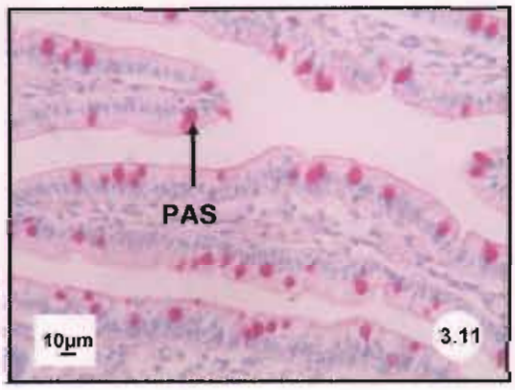

Figure 3.11: DPAS (magenta) staining of mucous cells in rat ileum (x400 digital camera).

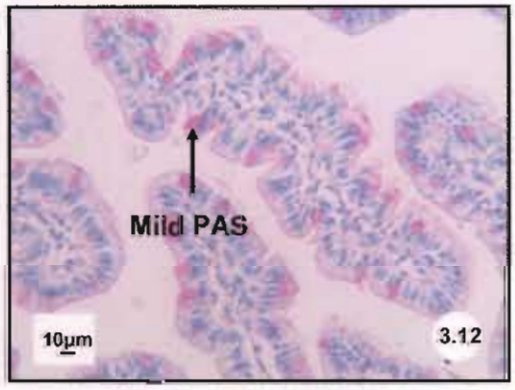

Figure 3.12: Mild PAS (pink) staining of mucous cells in rat ileum (x400 digital camera).

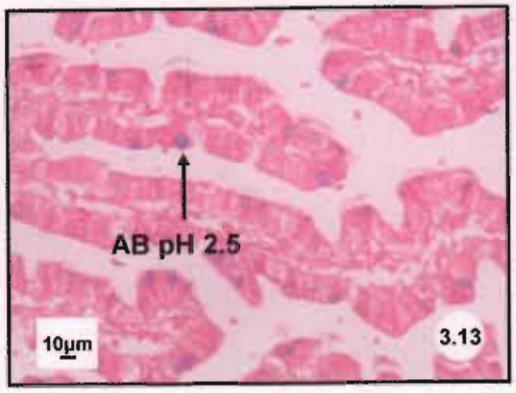

Figure 3.13: AB (pH 2.5) (blue) staining of mucous cells in rat ileum (x400 digital camera).

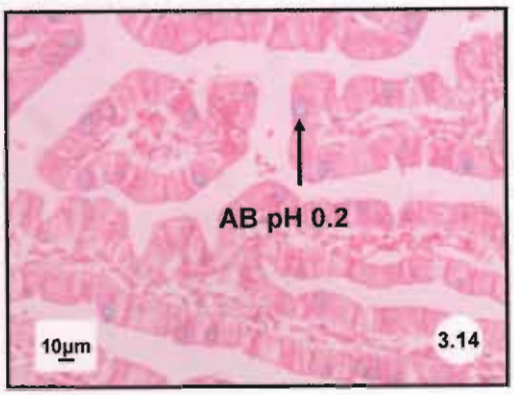

Figure 3.14: AB (pH 0.2) (blue) staining of mucous cells in rat ileum (x400 digital camera).

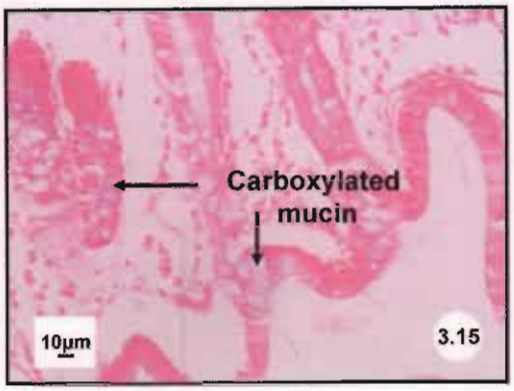

Figure 3.15: Methylation-saponification staining of carboxylated mucins (blue) in rat ileum (x400 digital camera).

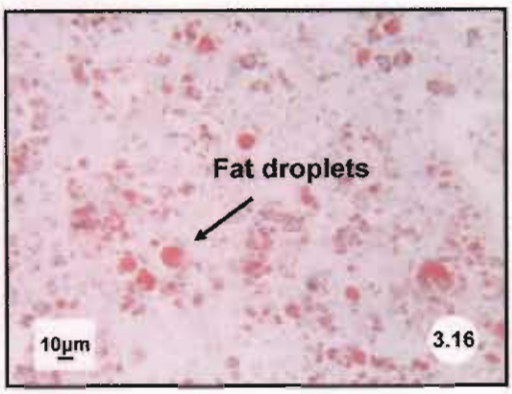

Figure 3.16: ORO (red) staining of fat control (x400 digital camera).

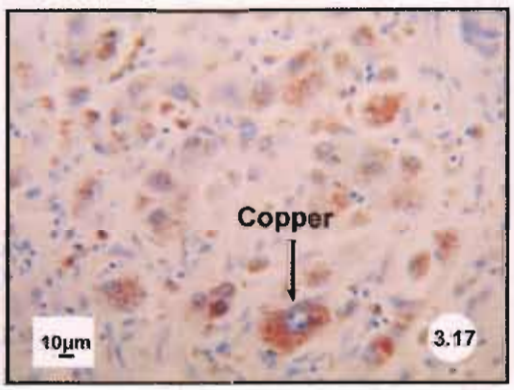

Figure 3.17: Rhodanine technique (red/orange) staining copper in rat liver (x400 digital camera).

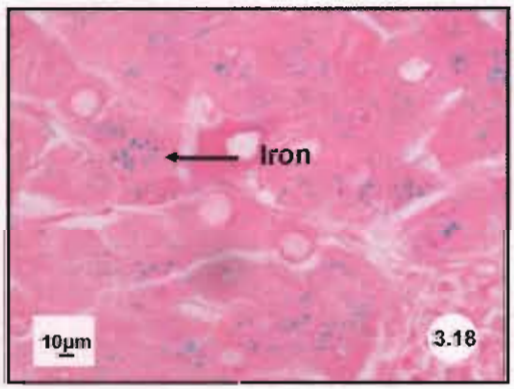

Figure 3.18: Perl's Prussian blue (blue) staining of iron granules in rat liver (x400 digital camera).

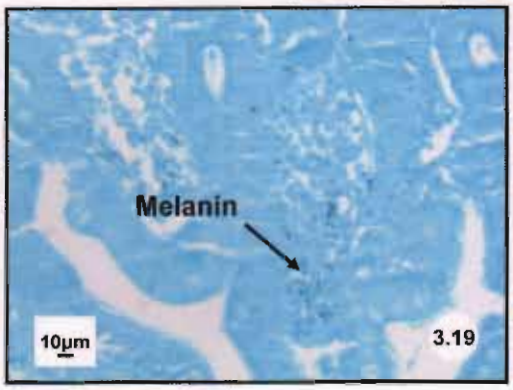

Figure 3.19: Masson Fontana (black) staining melanin in rat intestine (x400 digital camera).

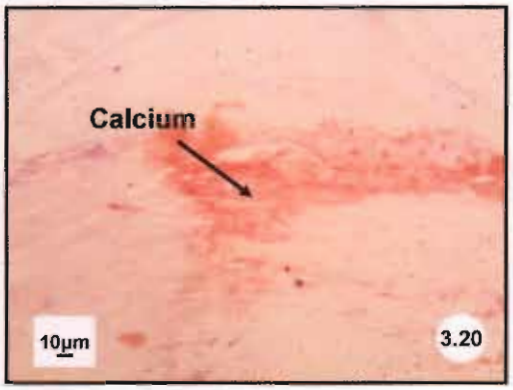

Figure 3.20: Alizarin Red S (red) staining calcium in rat kidney (x400 digital camera).

APPENDIX E: CHAPTER 4 SOLUTIONS

TE BUFFER

10 mM Tris.Cl

1 mM EDTA

SOC MEDIUM (pH 7 and Autoclaved)

20 g Tryptone

5 g Bacto yeast extract

0.5 g NaCl

10 ml 250 mM KCl

5 ml 2 M $MgCl_2$

20 ml 1 M Glucose

Make up to 1 L with distilled water.

LB AGAR (Autoclaved)

10 g Tryptone

5 g Yeast extract

10 g NaCl

15 g Agar

Make up to 1 L with distilled water

X-GAL

40 mg/ml in DMF

LB BROTH (Autoclaved)

10 g Tryptone

5 g Yeast extract

10 g NaCl

Make up to 1 L with distilled water.

SOLUTION I

50 mM Glucose

25 mM Tris.Cl (pH 8)

10 mM EDTA (pH 8)

SOLUTION II

0.2 M NaOH

10% SDS

SOLUTION III

5 M Potassium acetate	60 ml
Glacial Acetic acid	11.5 ml
Distilled H_2O	28.5 ml

PUBLICATIONS AND PRESENTATIONS

POSTER PRESENTATIONS

- Analysis of enzyme, carbohydrate and mineral distribution in the foot of abalone shellfish. **98th Annual Meeting of the National Shellfisheries Association, Monterey, California, March 26-30, 2006.**
- Evaluation of Cell Proliferation by Immunocytochemistry in the Shellfish Abalone (*Haliotis* spp.). ***BioNet*** **Annual Conference 2003, 16th-17th December, Galway-Mayo Institute of Technology, Ireland.**

ORAL PRESENTATIONS

- Development of PCR techniques for the detection of *Vibrio carchariae* and *Perkinsus olseni* in abalone tissues. **98th Annual Meeting of the National Shellfisheries Association, Monterey, California, March 26-30, 2006.**

PUBLICATIONS

- Harris, L., Lambkin, H. and O'Byrne-Ring, N. 2006. Characterisation of cell types in abalone (*Haliotis* spp.) tissues using immunohistochemical techniques. *Aquaculture*. **261**, 1413-1421.
- Harris, L., Lambkin, H. and O'Byrne-Ring, N. 2006. Analysis of enzyme, carbohydrate and mineral distribution in the foot of abalone shellfish. *J. Shellfish Res*. **25**, 736-737.
- Harris, L., Ryan, F., Lambkin, H. and O'Byrne-Ring, N. 2006. Development of PCR techniques for the detection of *Vibrio carchariae* and *Perkinsus olseni* in abalone tissues. *J. Shellfish Res*. **25**, 737.

- Molecular detection of *Vibrio* spp. infection in the abalone *Haliotis tuberculata* by multiplex PCR. **In submission to *Diseases of Aquatic Organisms*.**
- Analysis of functional enzymes in abalone shellfish (*Haliotis tuberculata* and *Haliotis discus hannai*) by Histochemistry. **Draft in progress.**

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Characterisation of cell types in abalone (*Haliotis* spp.) tissues using immunohistochemical techniques

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Abstract

The increasing popularity of abalone as a seafood delicacy has led to the rapid development of abalone aquaculture worldwide. The commercial and economic importance of this industry has resulted in an increasing interest in the biology of this particular shellfish genus. In this study we focus on the identification of structural, functional and proliferative proteins in two species of abalone shellfish, *Haliotis discus hannai* and *Haliotis tuberculata*. Monoclonal and polyclonal antibodies that react with proteins in vertebrate and invertebrate tissues were selected and applied to abalone tissues. Cross sections of whole animals were analysed using avidin–biotin immunoperoxidase staining protocols. In total, twenty-four antibodies were tested on shellfish tissues. Six antibodies out of twenty-four detected antigens in *Haliotis* spp. Cytokeratins, proliferating cell nuclear antigen (PCNA), neuron specific enolase (NSE), laminin and vimentin were detected in abalone tissues. Positive immunohistochemical results were confirmed using western blot. The expression of these proteins aids in the characterisation of cell types present in abalone tissues, which contributes to a better understanding of the fundamental biology of this shellfish genus.

Keywords: Abalone; Cell markers; *Haliotis tuberculata*; *Haliotis discus hannai*; Immunohistochemistry

1. Introduction

Abalone are marine gastropods consisting of 56 described species found in both temperate and tropical waters of both hemispheres (Bevelander, 1988; Geiger, 2000). Abalone aquaculture has become an economically important activity worldwide owing to a significant increase in consumer demand for this shellfish product. World cultured abalone production has soared phenomenally in recent times with over fifteen species currently in commercial cultivation (Gordon and Cook, 2004). Abalone shellfish are not native to Ireland but they were introduced in 1970. Land-based farms were set up along the Western seaboard for the commercial cultivation of two particular species of abalone, *Haliotis tuberculata* and *Haliotis discus hannai*. The histology of prosobranch and abalone shellfish has been studied (Bevelander, 1988; Voltzow, 1994) but there is a scarcity of information on the cellular and molecular constituents of shellfish in general.

Immunohistochemistry is a technique that demonstrates phenotypic antigen expression and was developed in the early 20th century. It allows for the demonstration of antigens in tissue sections by the use of specific immunological (antibody–antigen) interactions culminating in the attachment of a visible marker

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(usually an enzyme) to the antigen (Avrameas and Uriel, 1966; Nakane and Pierce, 1966). Cell markers are proteins characteristic of certain cell types. They may arise as surface cell markers that are molecules or proteins characteristic of the plasma membrane of a cell or as intracellular proteins that occur within the cell. Cell types and tissues express cell specific markers, which can be identified through immunohistochemistry (Miller, 2002).

The characterisation of cell types in higher vertebrates has been researched extensively but the information available for invertebrates remains scarce (Bacetti et al., 1984; Lyons-Alcantara et al., 1999). The human antibody repertoire can collectively target thousands of antigens but there is currently a limited range of antibodies that react with invertebrate antigens (Vullings et al., 1989; Lyons-Alcantara et al., 1999, 2002; Panasophonkul et al., 2004). Owing to this factor immunohistochemical techniques have rarely been employed in the study of shellfish but there are a few studies that have utilised immunolocalisation methods. In marine research the use of monoclonal antibodies and immunohistochemical techniques has been concentrated in the study of marine immunology and infectious disease (Yoshino and Granath, 1983; Mialhe et al., 1988; Noël et al., 1994; Coll and Dominguez-Juncal, 1995; Roch, 1999; Xue and Renault, 2001). More recently immunohistochemical methods, using rabbit and mouse polyclonal antibodies, were employed to locate serotonergic and FMRF-amidergic neurons within the cerebral, pleuropedal and visceral ganglia of *Haliotis asinina* (Panasophonkul et al., 2004).

Immunohistochemistry has also been used in the study of cytokeratins in fish and invertebrates (Markl and Franke, 1988; Markl et al., 1989; Bunton, 1993; Diogo et al., 1994). Cells of the digestive gland of the Norwegian prawn, *Nephrops norvegicus*, were characterised using immunohistochemistry (Lyons-Alcantara et al., 1999) and the localisation of the nuclear protein PCNA within the nuclei of both fish and prawns was also established using this technique (Suzuka et al., 1989; Ortego et al., 1994).

This research concentrates on the evaluation of antigen expression and protein typing in shellfish tissues. It has been suggested that all vertebrate and invertebrate intermediate filament proteins share a common antigenic determinant (Pruss et al., 1981). Homologues of vertebrate type I, II and III intermediate filament proteins were identified in the invertebrate *Branchiostoma lanceolata* (Karabinos et al., 1998). It is evident that some proteins are highly conserved in evolution due to the cross reaction of antibodies to human antigens with epitopes in shellfish tissues. This study focuses on the immunohistochemical evaluation of structural and functional proteins in shellfish tissues that have not been previously characterised. A wide range of antibodies to fish and human antigens are investigated to determine their cross reactivity with similar proteins present in abalone tissues using

Table 1
Specifications and controls for antibodies that bind with antigens in human/mammalian species

Antibody*	Source of immunogen	Clone	Optimal dilution	Antigen retrieval	Positive control
CD† 20	Human	L26	1:1200	Microwave	Tonsil
CD† 34	Human	QBEnd 10	1:50	Microwave	Tonsil
CD† 68	Human	KP1	1:50	Microwave	Tonsil
Chromogranin A	Human	DAK-AE	1:500	Microwave	Ileum
Cytokeratin AE1/3	Human	AE1/3	1:800	Protease	Pancreas
Cytokeratin 8	Human	4.1.18	1:50	Microwave and protease	Pancreas
Ki67	Human	MIB-1	1:25	Microwave	Tonsil
Cytokeratin MNF 116	Human	MNF 116	1:120	Protease	Ileum
Collagen IV	Human	CIV22	1:450	Microwave	Kidney
Human Desmin	Human	D 33	1:50	Microwave	Intestine
34βE12	Human	34 βE12	1:20	Protease	Pancreas
LP 34	Human	LP34	1:50	Protease	Skin
Muscle Specific Actin (MSA)	Human	HHF 35	1:100	Microwave	Pancreas
Neurofilament	Porcine	2F 11	1:50	Microwave	Cerebellum
Neuron Specific Enolase	Human	BB/NC/VI-H14	1:600	Microwave	Intestine
S100	Bovine	S100	1:400	Microwave	Skin/Sal gland
Synaptohysin	Bovine	SY38	1:20	Microwave	Pancreas
Vimentin	Bovine	VIM 3B4	1:50	Microwave	Tonsil
Von Willebrand Factor	Human	F8/86	1:75	Microwave	Tonsil

*All antibodies used were supplied by DakoCytomation™, Galway, Ireland.
†CD — Cluster of Differentiation.

Table 2
Specifications and controls for antibodies that bind with antigens in both vertebrate and invertebrate species

Antibody	Source of immunogen	Clone	Supplier	Optimal dilution	Antigen retrieval	Positive control
Cytokeratin AE1	Human	AE1	Serotec	1:100	Protease	Pancreas
PCNA	Rat	PC10	Serotec	Neat	Microwave	Tonsil
Vimentin	Porcine	V9	Serotec	1:80	Microwave	Smooth muscle
Neurofilament	Rat	RmdO-20	Zymed	1:50	Microwave	Cerebellum
Laminin	Mouse	Polyclonal	Abcam	1:100	Protease	Liver

immunohistochemistry. Where positive immunohistochemical results are observed, western blot analysis is employed to confirm the presence of these specific proteins in abalone tissues.

2. Materials and methods

2.1. Specimen collection and processing

H. tuberculata and *H. discus hannai* specimens were obtained from the Boet Mór shellfish farm in Clifden, Co. Galway, Ireland. The animals ranged from one to three years old.

Animals were anaesthetised in a solution of alcohol and seawater (1:1) for 30 min and transferred to 100% absolute alcohol for 30 min. The shells were removed and the animals were placed in Davidson's fixative for 24 h. An incision was made lengthwise down the foot muscle of each mollusc to allow penetration of the fixative.

Individual organs were dissected from shellfish 5 cm in length and animals less than 2 cm in length were cross-sectioned longitudinally.

2.2. Paraffin sections

All animals were processed through the following solutions: 10% formalin, spirit (95% ETOH), absolute alcohol (×5), xylene (×3) and paraffin wax (×2). Tissues were embedded in paraffin wax and 5 μm sections were cut using a microtome. Sections were set onto adhesive APES (3-aminopropyltriethoxysilane) coated slides and incubated at 56 °C for 2–3 h. The following antigen retrieval methods were used on some tissues to enhance immunostaining: (a). microwaving: dewaxed and rehydrated tissue sections were placed in 500 ml citrate buffer (2.1 g citric acid in 1 l of distilled water at pH 6 using 2 M NaOH) microwaved (800 W) for 18 min and incubated in buffer for a further 20 min and (b). proteolytic digestion: dewaxed and rehydrated tissue sections were incubated in 0.1% protease (*Streptomyces griseus*, Type 24, Sigma) in PBS for 10 min at 37 °C.

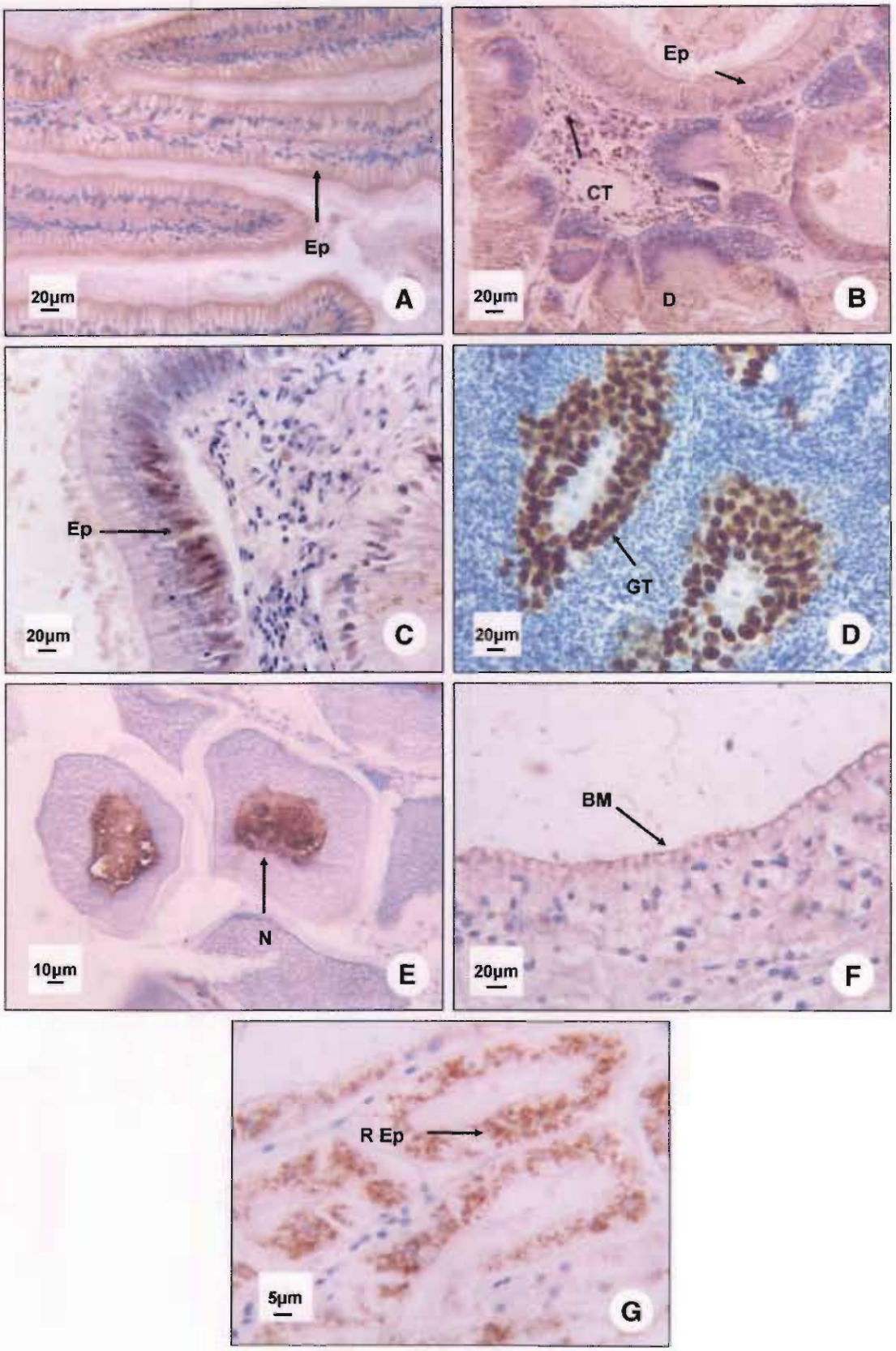
2.3. Immunostaining protocol

Shellfish and human tissues were stained using the Vectastain® Elite Avidin–Biotin Complex kits. After antigen retrieval the slides were treated with 3% hydrogen peroxide in methanol for 10 min. Following rinsing in water, sections were incubated in PBS for 5 min. Sections were covered with normal horse serum (1:100 universal kit; 1:67 mouse kit) for 5 min. They were then drained and the primary antibody diluted in PBS was applied to sections for 60 min (Tables 1 and 2). The slides were rinsed in PBS (×3) and incubated in biotinylated secondary antibody (1:25 universal kit; 1:50 mouse kit) for 15 min. Slides were washed in 3 changes of PBS buffer and incubated for 15 min in avidin–biotin complex reagent (1:25 with both kits). Sections were rinsed in buffer and the chromogen, diaminobenzidine-peroxide (0.06 mg ml^{-1} with 0.03% hydrogen peroxide) was applied for 5 min to produce a brown reaction product. Slides were counterstained in Mayer's Haematoxylin for 1 min and dehydrated

Table 3
Specifications and controls for antibodies used in western blotting

Antibody	Source of immunogen	Clone	Supplier	Optimal dilution	Positive cell line control	Molecular weight (kDa)
Cytokeratin AE1	Human	AE1	Serotec	1:100	HeLa	40; 48; 50; 56
Cytokeratin MNF	Human	MNF 116	DAKO	1:120	HeLa	45–56.5
PCNA	Rat	PC10	Serotec	Neat	HeLa	36
Vimentin	Porcine	V9	Serotec	1:80	CHO[a]	57

[a] CHO — Chinese Hamster Ovary cell line.


Ep
20μm
A
Ep
CT
20μm
D
B
Ep
C
GT
20μm
D
N
10μm
E
BM
20μm
F
R Ep
5μm
G

through graded alcohols, cleared in xylene and mounted in Distrene Plasticiser Xylene (DPX). Human tissues were used as positive controls as listed in Tables 1 and 2. Human tissues used as positive controls were supplied by other DIT ethically approved projects. For negative controls, all reagents were applied to abalone tissues except the primary antibody.

2.4. Protein extraction

Human cell lines were grown in culture and protein was extracted from fully confluent cells which served as positive controls in western blotting experiments (Table 3). Protein was extracted from cell lines using NP40 Triple detergent lysis buffer (0.1% SDS; 150 mM NaCl; 50 mM Tris–Cl (pH 8.0); 1% Nonidet P-40 (NP-40)). 3 ml of NP40 lysis buffer was added to a flask of confluent cells. The lysis buffer containing the cells was centrifuged at 13,000 rpm for 20 min at 4 °C and the supernatant was stored at −20 °C. For protein extraction from abalone tissues, approximately 200 mg of fresh frozen tissue was macerated and suspended in 2 ml SDS lysis buffer (2% SDS; 50 mM Tris–HCl (pH 7.2); 1 mM β-mercaptoethanol) and boiled for 3 min (Cummins and Hanna, 2004). The sample was homogenised and boiled for 5 min. Samples were centrifuged at 13,000 rpm for 10 min and the supernatant was collected for storage at −20 °C. Soluble protein concentrations (μg/ml) of human cell line extracts and abalone tissue extracts were analysed using the Bradford protein assay.

2.5. Western blot analysis

For western blot analysis proteins separated by SDS-PAGE analysis were transferred to a PVDF (polyvinylidene difluoride) membrane using a semi-dry western blotter (Apollo™ Instrumentation). The blotter was run at 2 mA per cm^2 of gel for 2 h after which the membrane was removed and washed briefly with TBS (Tris Buffered Saline) buffer. The membrane was incubated in 5% BSA blocking solution overnight at 4 °C on a shaker at a low setting. After blocking the membrane was washed briefly in TBS and the primary antibody was added to the membrane at the appropriate dilution in 5% BSA blocking solution (Table 3). The membrane was incubated in primary antibody at room temperature for 2 h on a shaker. The membrane was washed in 5% BSA blocking solution (×3) for 15 min. The biotinylated secondary antibody (diluted in 5% BSA blocking solution) was added and the membrane was incubated for 1 h at RT. The membrane was washed in 5% BSA blocking solution (×3) for 15 min. The ABC reagent (diluted in 5% BSA blocking solution) was added and the membrane was incubated for 1 h at RT. The membrane was washed in 5% BSA blocking solution (×3) for 15 min. The membrane was finally incubated in DAB (0.06 mg ml^{-1} in PBS with 0.03% hydrogen peroxide) to produce a brown reaction product for the protein of interest. The membrane was washed in 5% BSA blocking solution and allowed to air dry prior to storage in the dark.

3. Results

3.1. Application of antibodies that bind with human/mammalian antigens to abalone tissues

Nineteen antibodies that bind to mammalian antigens were applied to abalone tissues (Table 1). Cross sections of whole animals were used in preliminary trials and any positive staining was subsequently verified by staining individual shellfish organs. Two out of the nineteen antibodies tested reacted with antigens in abalone tissues: cytokeratin MNF 116 and NSE (Fig. 1A and B). All human positive control tissues were positive for each antibody tested and negative controls were negative (Table 1). A brown reaction product indicates a positive reaction.

Cytokeratin MNF expression was concentrated in the epithelial cells of the intestine of abalone. The surfaces of the epithelial cells were labelled for this antibody (Fig. 1A). Other epithelial cells of the digestive tract also stained positively for MNF but a more intense intestinal epithelial expression was observed.

NSE produced a pattern of positive staining in the epithelia of the digestive system. This antibody is directed against a neural protein. Antigens within the epithelial cells of the digestive tract and in the duct cells of the hepatopancreas stained positively with this antibody. NSE positive cells were also observed within the connective tissue of the digestive tract (Fig. 1B).

Fig. 1. A. Epithelial cells (Ep) of the intestine of *Haliotis discus hannai* positive for cytokeratin MNF (brown). B. Duct cells (D), epithelial cells (Ep) and connective tissue (CT) of the digestive gland of *Haliotis discus hannai* positive for NSE (brown). C. Epithelial cells (Ep) of the intestine of *Haliotis discus hannai* positive for cytokeratin AE1 (brown). D. Nuclei in the male gonadal tubules (GT) of *Haliotis discus hannai* positive for PCNA (brown). E. Nuclei (N) of the female eggs of *Haliotis discus hannai* positive for PCNA (brown). F. Basement membrane (BM) of the foot of *Haliotis discus hannai* positive for laminin (brown). G. Right renal epithelial cells (REp) of *Haliotis tuberculata* positive for vimentin (brown).

3.2. Application of antibodies that bind with other vertebrate/invertebrate antigens to abalone tissues

Five antibodies that bind to both vertebrate and invertebrate antigens were tested on cross sections of abalone tissues (Table 2). A positive result was then verified by staining sections from individual organs such as the digestive gland, epipodium, foot, gonad and kidney. All human positive control tissues were positive for each antibody tested and negative controls were uniformly negative (Table 2). Cytokeratin AE1 was expressed in some epithelial cells of the digestive system of abalone tissues but not in all epithelial cells. The positive reaction observed was not uniform rather scattered throughout the cells of the intestine (Fig. 1C). This cytokeratin was also expressed in the pedal epithelia of some abalone.

In abalone tissues both the male and female gonads expressed PCNA. PCNA positive cells were concentrated in the gonadal tubules of the reproductive organs of the male abalone. The cells of the gonad were strongly positive for the presence of this protein (Fig. 1D). Epithelial cells of the digestive system were also positive when stained with this antibody. The nuclei of large but immature ova in the female gonad were also PCNA positive (Fig. 1E).

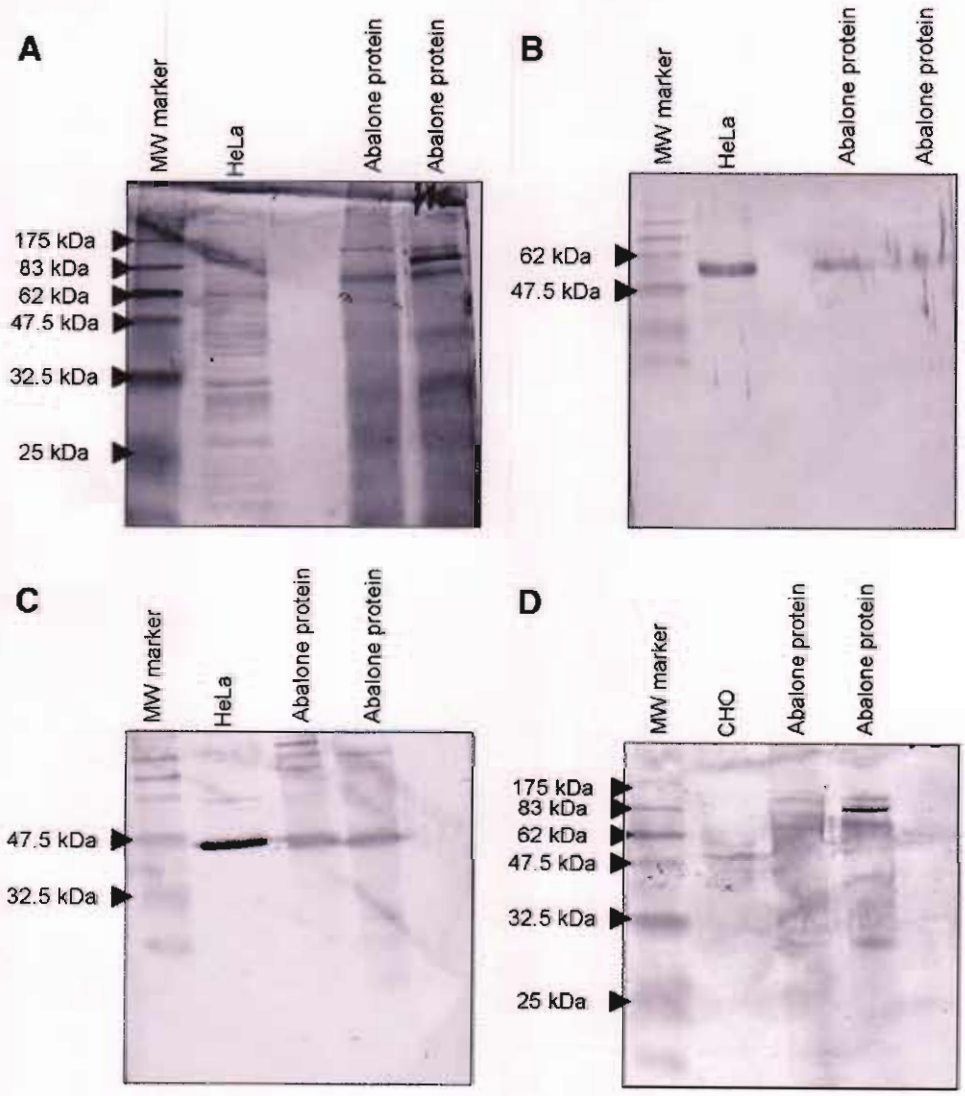


Fig. 2. A. Western blot of HeLa cells (11.3 μg) and abalone (*H. discus hannai* and *H. tuberculata*) tissue extracts (6.3 μg; 7 μg) using cytokeratin MNF 116 (DAKO). B. Western blot of HeLa cells (11.3 μg) and abalone (*H. discus hannai* and *H. tuberculata*) tissue extracts (6.3 μg; 7 μg) using cytokeratin AE1 (Serotec). C. Western blot analysis of HeLa cells (11.3 μg) and abalone (*H. discus hannai* and *H. tuberculata*) tissue extracts (6.3 μg; 7 μg) using PCNA (Serotec). D. Western blot of CHO cells (8.1 μg) and abalone (*H. discus hannai* and *H. tuberculata*) tissue extracts (6.3 μg; 7 μg) using vimentin V9 (Serotec).

The antibody laminin was expressed in the basement membrane below the pedal epithelial layer of the abalone foot (Fig. 1F).

Another clone of vimentin was used in this section of the study. A granular staining pattern was expressed in the cytoplasm of the right renal organ of abalone using this antibody (Fig. 1G).

3.3. Western blotting results

Proteins identified in abalone tissues by immunohistochemistry were further investigated and identified by ABC/DAB detection in western blotting using SDS-PAGE and transfer to PVDF membranes. Western blot analysis confirmed the presence of cytokeratin MNF, cytokeratin AE1, PCNA and vimentin in abalone tissues.

Western blot analysis confirmed the presence of cytokeratins in abalone using MNF and AE1 antibodies. The broad spectrum antibody cytokeratin MNF which detects a wide range of proteins, revealed proteins with molecular weights of between 25 and 175 kDa in the HeLa positive control (Fig. 2A). With abalone samples, 3 strong bands with molecular weights of 100, 83 and 70 kDa approximately were identified and weaker bands were observed in the 32.5–47.5 kDa range (Fig. 2A). A very strong band at 83 kDa in the second sample can be observed in Fig. 2A. The lower molecular weight bands correspond to keratins 5, 6, 8, 17 and 19 while the high molecular weight proteins identified do not correspond to known cytokeratins.

The antibody cytokeratin AE1 identifies proteins with molecular weights of 40–56.5 kDa. A single band with a molecular weight of 56.5 kDa approximately was identified in both the positive HeLa control cells and abalone samples, which corresponds to cytokeratin 10 (Fig. 2B).

Western blot analysis with PCNA identified clear bands with a molecular weight of approximately 36 kDa in the HeLa positive control and whole protein extracted from abalone tissues (Fig. 2C). In Fig. 2C specific bands between 83 and 175 kDa can be observed despite a lower concentration of protein than the positive control.

Stronger bands observed with HeLa positive controls compared with abalone protein in western blots with antibodies AE1 and PCNA, could be indicative of higher concentrations of extracted protein.

Protein bands were detected in abalone tissues with the vimentin antibody using western blotting (Fig. 2D). A band was observed in the CHO positive control which corresponds to the 57 kDa band expected with vimentin. A very strong band and two weaker bands were identified with molecular weights of between 83 and 175 kDa in the second abalone sample while the stronger band was not observed in the first sample. The strong band observed in Fig. 2D is very specific despite lower concentrations of abalone compared to the CHO positive control.

4. Discussion

Immunohistochemistry is a highly valued technique that allows for the study of functional and structural entities within tissues. It allows for the analysis of protein distribution within tissues and has the potential for application in marine research. However, the lack of specific antibodies to shellfish antigens remains a major limitation.

Previous studies have used immunohistochemistry to study the hemocytes/immune cells of molluscs (Yoshino and Granath, 1983; Ottaviani, 1989; Noël et al., 1994; Xue and Renault, 2001). These studies focused on the definition of molluscan hemocyte type and the determination of hemocyte distribution within tissues using antibodies produced specifically to detect these cells. Few if any shellfish studies are available that have aimed to characterise cells other than immune cells.

This study demonstrated cytokeratin expression in abalone tissues using broad spectrum antibodies MNF 116 and cytokeratin AE1. Keratins are intermediate filaments of epithelial cells and they have a structural role in tissues. The paper of Diogo et al. (1994) was the first study to characterise invertebrate cytokeratins. Since then cytokeratins have been characterised in the prawn and shrimp (Lyons-Alcantara et al., 1999, 2002). Cytokeratin MNF recognises proteins of molecular weight 45–56.5 kD and cytokeratin AE1 recognises proteins with a molecular weight of 56.5, 50, 48 and 40 kD. The epitopes that are thus being targeted by these two antibodies are in the same molecular weight range. Both antibodies react positively to normal human epithelia and their neoplasms showing a broad range of reactivity (Leong et al., 2002). Four cytokeratins were tested, and two were expressed in abalone tissues. Cytokeratin AE1 reacted positively with cytoplasmic proteins in the intestinal epithelia of abalone and cytokeratin MNF was present on the surface of the intestinal epithelia. These results were reinforced by western blot analysis and while AE1 produced a specific band of 56.5 kDa in abalone samples, western blotting with cytokeratin MNF revealed many protein bands in abalone tissues indicating numerous cytokeratins, some of which were very distinct.

PCNA is a multifunctional cell marker representing a component of DNA polymerase-δ. It is a proliferating cell marker producing a staining pattern that is generally confined to the nuclei of actively proliferating tissues and cells. This cell marker has been detected in mammals, prawns, fish and some higher plants (Suzuka et al., 1989; Ortego et al., 1994; Lyons-Alcantara et al., 1999). In this study PCNA was detected in abalone tissues. Antigens in the nuclei of the reproductive organs of both male and female abalone reacted positively with this antibody. Cells of the gonad are constantly undergoing proliferation giving rise to new cells thus explaining the presence of PCNA in these areas. Not all abalone proliferating cells expressed this antigen, mainly epithelial proliferating cells. PCNA positivity was also located in the nuclei of epithelial cells of the hepatopancreas, however the degree of positivity within epithelial tissues was scattered. Western blot analysis also confirmed the presence of PCNA in abalone tissues and additional molecular weight bands, higher than the 36 kDa specific band were observed.

Vimentin, a protein expressed in virtually all mesenchymal cells was also present in abalone tissues. Vimentin has been shown to produce a cytoplasmic pattern of staining in the invertebrate *Drosophila melanogaster* (Walter and Biessmann, 1984) and has been recognised as a cytoskeletal constituent of many fish tissues (Nelson and Traub, 1982; Gyoeva et al., 1987) and in invertebrate tissues (Karabinos et al., 1998). Markl et al. (1989) used gel electrophoresis, immunostaining and immunoblot assays to identify vimentin in rainbow trout tissues. They concluded that the identified cytoskeletal protein is homologous to mammalian vimentin but it only makes up a very small component of the cytoskeleton. In this study the V9 clone (57 kDa) detected vimentin in the kidney of abalone and a granular cytoplasmic staining pattern was observed which was confirmed by western blotting with bands in the 57 kDa range but more definite bands were observed at 83 kDa.

Three different antibodies were employed to detect elements of the nervous system of abalone, NSE, neurofilament and S100. Neurofilament and S100 were not detected while NSE, an isoenzyme of enolase present in neurons and neuroendocrine cells (Leong et al., 2002) was demonstrated in the epithelial cells of the digestive gland and in the connective tissue of the digestive tract. The prosobranch body is entirely innervated and NSE could be cross-reacting with an enolase enzyme released by the intestinal ganglia. Western blot analysis did not confirm the presence of NSE in abalone tissues. Nerve cells have recently been characterised in *H. asinina* Linnaeus using antibodies to serotonin and FMRF-amide neurotransmitters (Panasophonkul et al., 2004).

Another antibody that cross-reacted with antigens in abalone tissues was laminin. Its presence indicates the highly conserved nature of this structural protein, being present in many species from invertebrates to vertebrates (Sarras et al., 1994; Zhang et al., 1994; Yurchenco and Waddsworth, 2004). Laminin is an embryonically expressed protein that is essential for basement membrane assembly and is one of the most ancient proteins within extracellular matrices (Cooper and McQueen, 1983; McCarthy et al., 1987; Montell and Goodman, 1989; Yurchenco and Waddsworth, 2004). Previous studies on invertebrate laminins have employed immunofluorescent and immunohistochemical techniques to identify these proteins (Sarras et al., 1993, 1994). Laminin was observed in the basement membrane of the abalone foot. Western blot analysis was not performed with laminin since only a small number of basement membranes expressed this protein with immunostaining.

The use of mammalian and veterinary antibodies in this study gives an indication of the structural, functional and proliferative framework that makes up the tissues of abalone, which will be helpful in studying the effects of pathogens on this shellfish through comparisons of both healthy and diseased organisms. The key findings of this study are that PCNA, cytokeratins, NSE and vimentin are important biomarkers of proliferation and differentiation in abalone species. Immunohistochemistry acts as a helpful adjunct to histology, histochemistry and other studies of cellular function. However further progress in this area is dependent upon antibodies specific to shellfish antigens being developed.

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TROPHIC CONSEQUENCES OF A LONG-LIVED NONNATIVE PREDATOR (*RAPANA VENOSA*) ON ESTUARINE COMMUNITY DYNAMICS. **Juliana M. Harding** and **Roger Mann.** VIMS, P.O. Box 1346, Gloucester Point, Virginia, USA 23062.

Veined rapa whelks, *Rapana venosa,* are large generalist predators with the potential to live in excess of 10 yrs and life history suitable for successful invasion of estuarine habitats. The presence of rapa whelks in a habitat has obvious consequences for the prey field in that rapa whelk prey consumption shifts ontogenetically from small (e.g., *Mytilus* sp., *Macoma* sp, *Mya* sp, *Crassostrea* sp.) to large (e.g. *Mercenaria* sp, *Crassostrea* sp.) prey. Laboratory and mesocosm experiments indicate that wild rapa whelks reach a size refuge from predation by blue crabs (*Callinectes sapidus*) within one year post settlement at critical sizes of 30–40 mm SL. At sizes >40 mm, rapa whelk distribution in Chesapeake Bay is probably limited only by salinity and food availability. The presence of large predators with the ability to repel competitors (blue crabs) on shared feeding grounds may force native species into habitat refugia delimited by salinity tolerances. Scenarios predicting relative abundance and competitive interactions between bivalve prey, rapa whelks, and blue crabs with impacts on trophic dynamics and habitat use are discussed.

GENETIC TRACKING OF RESTORATION OYSTERS TO GAUGE SUCCESS—A COST/BENEFIT ANALYSIS. **Matthew P. Hare,** University of Maryland.

Restoration of oyster stocks in Chesapeake Bay is a monumental task to which abundant resources have been committed and for which many dedicated parties have labored. Beyond establishing a ten-year goal for the increase of census numbers, little attention was initially paid to establishing rigorous benchmarks for success, or collecting data that could either measure small successes or determine the cause of failures. This has been changing more recently, but now most restoration effort is focused on targeted population supplementation using artificially selected, disease tolerant *C. virginica* to combat high mortalites from parasitic diseases. This tactic has potential benefits and considerable risks. I will argue that genetic testing of recruitment is necessary to evaluate and manage the genetic risks of supplementation with inbred oysters, but it also provides the most meaningful measure of overall restoration efficacy. Results will be presented from a highly collaborative effort to genetically monitor DEBY-strain restoration plantings in two Chesapeake subestuaries. The results indicate that the hatchery amplification of DEBY broodstock to produce restoration oysters is contributing to inbreeding in the oysters over and above that already realized during artificial selection. Also, the DEBY contribution to local recruitment in the Great Wicomico River in 2002 was approximately 10%, a result that belies appearances based on the number of DEBY oysters planted and the elevated levels of recruitment that year. Inbreeding may or may not be the greatest risk facing restoration oysters and threatening restoration success, but only improved genetic testing will allow informed risk management.

METABOLIC RATES OF *CRASSOSTREA ARIAKENSIS* AND *CRASSOSTREA VIRGINICA* AT TWO TEMPERATURES AND THREE SALINITIES. **Nicole Harlan, Kennedy Paynter** and **Donald Meritt.** University of Maryland Center for Environmental Science.

Maryland and Virginia have proposed to replace the native oyster, *Crassostrea virginica,* with the suminoe oyster, *Crassostrea ariakensis,* in Chesapeake Bay. *C. virginica,* is highly tolerant of hypoxic conditions and can survive emersion or nearly anoxic seawater for days to weeks depending on the temperature. In order replace *C. virginica*'s ecological niche of establishing vast benthic reefs in Chesapeake Bay, *C. ariakensis* may require similar tolerances. However, when the oysters were placed in sealed jars of anoxic water, *C. ariakensis* lived for an average of four days, while *C. virginica* persisted for more than fourteen days. Studies at 22°C have shown that the metabolic rate of *C. ariakensis* (1.96 ± 0.102 O_2/hr/gdw) is significantly higher than that of *C. virginica* (1.15 ± 0.079 mg O_2/hr/gdw; $p = 0.0244$). In order to better understand the aerobic requirements of these two species under different conditions, standard metabolic rates of each species were determined at two temperatures, 10 and 20°C, and three salinities, 5, 15, and 25 psu. At both temperatures and all three salinities, the metabolic rate of *C. ariakensis* was higher than that of *C. virginica.* Upon immersion in the test chambers, *C. ariakensis* gaped and began using oxygen within minutes, while *C. virginica* kept their valves shut for much longer. These data may influence the decision to use *C. ariakensis* as an ecological substitute for *C. virginica* in the Chesapeake Bay.

ANALYSIS OF ENZYME, CARBOHYDRATE AND MINERAL DISTRIBUTION IN THE FOOT OF ABALONE SHELLFISH. **Leanne Harris, Helen Lambkin** and **Nuala O'Byrne-ring.** Dublin Institute of Technology, Biological Sciences, Kevin Street, Dublin 8, Ireland.

The meat of the abalone shellfish has been labelled as a luxury food for thousands of years. The source of this epicurean delicacy is the foot, which is the most conspicuous external feature of this organism. The foot is a large muscular organ with an extensive nerve and vascular supply that serves both sensory and locomotory functions. The foot is also involved in many other functions such as locating and manipulating food, attaching eggs to substrates, cleaning the shell, finding potential mates and thwarting predators.

The foot is primarily made up of epithelial tissue, connective tissue and muscle. In this study the distribution of functional and structural elements was investigated in the pedal organ of two species of abalone, *Haliotis tuberculata* and *Haliotis discus hannai*. The pedal and peripheral epithelia expressed high levels of activity for the following enzymes: chloroacetate esterase, α-naphthyl butyrate esterase, alkaline and acid phosphatase, peroxidase and carbonic anhydrase. The sub-epithelial ganglion cells were positive for α-naphthyl butyrate esterase and acetylcholinesterase. Neutral mucins, acid mucins, carboxylated mucins and sulphated mucins were found in epithelial cells, in sub-epidermal gland cells and in the ground substance of the connective tissue and muscle. Melanin was identified in the sub-epidermal gland cells and in the pedal and peripheral epithelial cells. The basement membrane of the pedal epithelium was positive for calcium. A myriad of cell components and cellular activities in the tissues of the abalone foot were demonstrated, revealing cell types and reflecting the molecular pathways at work within these tissues.

DEVELOPMENT OF PCR TECHNIQUES FOR THE DETECTION OF *VIBRIO CARCHARIAE* AND *PERKINSUS OLSENI* IN ABALONE TISSUES. **Leanne Harris, Fergus Ryan, Helen Lambkin** and **Nuala O'Byrnering.** Dublin Institute of Technology, Biological Sciences, Kevin Street, Dublin 8, Ireland.

Global demand for abalone has significantly increased in recent years, however, wild stocks of abalone have declined in number, as a result of overexploitation and the spread of infectious disease. Disease outbreaks are recognised as a significant constraint to aquaculture production and trade that affects both economic development and the socio-economic revenue of many countries. Bacteria and protozoa are the most commonly encountered pathogens in abalone shellfish. *Vibrio harveyi/carchariae* is a highly pathogenic bacterium to abalone and *Perkinsus atlanticus/olseni* is a protozoan that also causes severe disease in this shellfish. Both pathogens are ranked amongst the top ten most significant disease causing organisms of abalone. In this study, a multiplex PCR method was developed to simultaneously amplify a 413 bp region of the 16S rRNA sequence of *V. carchariae/harveyi* and a 155 bp region of the actin mRNA gene sequence of *Haliotis spp.* This multiplex PCR was used to amplify these sequences in both fixed tissues and paraffin embedded tissues of infected *Haliotis tuberculata*. A primer set was designed to target a 245 bp region of the ITS sequence of *P. atlanticus* from paraffin embedded samples of infected *Ruditapes decussatus* which could be adapted to detect *P. olseni* in abalone tissues. Also quantitative PCR using these primers is a further potential development. These PCR protocols offer a rapid and specific method for the identification of *V. carchariae* and *P. olseni* in shellfish.

COMPARATIVE GROWTH AND SURVIVAL OF DIPLOID AND TRIPLOID SUMINOE OYSTERS, *CRASSOSTREA ARIAKENSIS*, IN MULTIPLE QUARANTINE SYSTEMS. **Heather D. Harwell** and **Standish K. Allen Jr.** Virginia Institute of Marine Science, P.O. Box 1346, Gloucester Point, VA 23062.

Much of the research on *Crassostrea ariakensis* has revealed superior growth rates and resistance to disease compared to the native oyster, *C. virginica*. All field studies of growth and survival have utilized sterile triploid oysters for reasons of biosecurity. Thus, triploids are serving as a surrogate for diploid performance in these field trials. A direct, simultaneous comparison of the growth and survival of diploid and triploid *C. ariakensis* is needed to refine population growth models based on triploid field data. Three replicate lines of diploid and triploid *C. ariakensis* were placed at four quarantine systems in Virginia and Maryland in December 2004. Individual repeated measures of subsets of oysters were gathered from monthly data on percent survival, wet weight, and shell length. In addition, quarterly estimates of condition index were obtained. Data gathered in this fashion will be used to determine a correction factor(s) that can then be applied to results from past studies of triploid *C. ariakensis* in order to refine models of potential population growth.

ROLE OF LOW SALINITY REFUGE IN REGULATING THE PREVALENCE OF THE PARASITE *LOXOTHYLACUS PANOPAEI* IN THE XANTHID *PANOPEUS OBESUS*. **Lesli Haynes[1], S. Gregory Tolley[1], Aswani K. Volety[1]** and **James T. Winstead[2].** [1]Florida Gulf Coast University, 10501 FGCU Blvd. S., Ft. Myers, FL 33965, [2]United States Environmental Protection Agency.

This study was conducted to examine the potential influence of salinity, a proxy for freshwater inflow, on the prevalence of the castrator parasite, *Loxothylacus panopaei*, on mud crabs found on Southwest Florida oyster reefs. Spatial and seasonal patterns of the presence of potential host crabs and the prevalence of the parasite were assessed in the Caloosahatchee, Estero, and Faka-Union estuaries of Southwest Florida. Lift nets (1 m^2) containing five liters of oyster clusters were deployed on intertidal reefs at three sites along the salinity gradient of each estuary. Nets were deployed during three seasonally dry and three seasonally wet months for a period of 30 d. Although *Panopeus obesus* were collected at all three locations within each estuary, densities tended to increase downstream in higher salinity waters. Parasite prevalence decreased at the upper stations in each estuary, was reduced during wet months compared to dry months, and was lower for those estuaries that experienced high levels of freshwater inflow. Furthermore, parasite prevalence was positively correlated with the mean salinity at capture of host crabs. Based on the distribution of *P. obesus* and the above patterns related to salinity, it appears that freshwater inflow and seasonal rains might regulate the prevalence